UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

Departamento de Sanidad Animal

TESIS DOCTORAL

Situación epidemiológica y clínica de la piroplasmosis equina en áreas endémicas como la Península Itálica e Ibérica

Epidemiological and clinical situation of equine piroplasmosis in endemic areas such as Italian and Iberian peninsulas /

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Leticia Elisa Bartolomé del Pino

Directores

Aránzazu Meana Mañes
Gian Luca Autorino

Madrid, 2017

ISBN: 978-84-09-05010-9

© Leticia Elisa Bartolomé del Pino, 2017

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

DEPARTAMENTO DE SANIDAD ANIMAL

SITUACIÓN EPIDEMIOLÓGICA Y CLÍNICA DE LA PIROPLASMOSIS EQUINA EN ÁREAS ENDÉMICAS COMO LAS PENÍNSULAS ITÁLICA E IBÉRICA

EPIDEMIOLOGICAL AND CLINICAL SITUATION OF EQUINE PIROPLASMOSIS IN ENDEMIC AREAS SUCH AS ITALIAN AND IBERIAN PENINSULAS

TESIS DOCTORAL

LETICIA ELISA BARTOLOMÉ DEL PINO

MADRID, 2017

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

DEPARTAMENTO DE SANIDAD ANIMAL

SITUACIÓN EPIDEMIOLÓGICA Y CLÍNICA DE LA PIROPLASMOSIS EQUINA EN ÁREAS ENDÉMICAS COMO LAS PENÍNSULAS ITÁLICA E IBÉRICA

EPIDEMIOLOGICAL AND CLINICAL SITUATION OF EQUINE PIROPLASMOSIS IN ENDEMIC AREAS SUCH AS ITALIAN AND IBERIAN PENINSULAS

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

LETICIA ELISA BARTOLOMÉ DEL PINO

DIRECTORES

ARÁNZAZU MEANA MAÑES
GIAN LUCA AUTORINO

MADRID, 2017

Leticia E. Bartolomé del Pino, 2017

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

DEPARTAMENTO DE SANIDAD ANIMAL

SITUACIÓN EPIDEMIOLÓGICA Y CLÍNICA DE LA PIROPLASMOSIS EQUINA EN ÁREAS ENDÉMICAS COMO LAS PENÍNSULAS ITÁLICA E IBÉRICA

EPIDEMIOLOGICAL AND CLINICAL SITUATION OF EQUINE PIROPLASMOSIS IN ENDEMIC AREAS SUCH AS ITALIAN AND IBERIAN PENINSULAS

TESIS DOCTORAL

LETICIA ELISA BARTOLOMÉ DEL PINO

MADRID, 2017

El trabajo presentado en esta tesis se ha realizado bajo la dirección de Gian Luca Autorino y Aránzazu Meana Mañes en el Centro de Referencia Nacional de las Enfermedades de los Équidos del Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana (IZSLT) en Roma, Italia con fondos del proyecto IZSLT 16/11 del Ministerio de Sanidad Italiano y en el Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid. Durante la realización del mismo la autora ha disfrutado de una beca Erasmus Prácticas concedida por la Universidad Complutense y una beca de apoyo a la investigación en cotutela ofrecida por el Ministerio de Asuntos Exteriores Italiano. Asimismo, se han realizado estancias en la Universidad Federico II de Nápoles (Italia).

Centro di Referenza Nazionale per le Malattie degli Equini, Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana - Roma (Italia) y
Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid.

Gian Luca Autorino, director del Centro di Referenza Nazionale per le Malattie degli Equini (CERME) dell'Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana - Roma (Italia) y M. Aránzazu Meana Mañes profesora titular del Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid.

CERTIFICAN:
Que la tesis doctoral «**Situación epidemiológica y clínica de la piroplasmosis equina en áreas endémicas como las Penínsulas Itálica e Ibérica.»** ha sido realizada por la licenciada en Veterinaria Dña. Leticia Elisa Bartolomé del Pino en ambos centros bajo nuestra dirección y estimamos que reúne los requisitos exigidos para optar al Título de Doctor por la Universidad Complutense de Madrid.

Gian Luca Autorino

M. Aránzazu Meana Mañes

Dedicada a mi familia y a quien se siente parte de ella

“C'est le temps que tu a perdu pour ta rose qui fait ta rose si importante.”
A. de Saint-Exupéry

AGRADECIMIENTOS

En primer lugar quiero agradecer a mis tutores Gian Luca Autorino y Aránzazu Meana la ayuda prestada, los consejos e ideas que me han ayudado a aprender a mirar la ciencia desde otro prisma.

Agradezco también a los técnicos y veterinarios del CERME del IZSLT de Roma el trato recibido durante este tiempo, a Teresa, Francesca, Roberto, Raffaele e Ida gracias por su disponibilidad, ayuda y paciencia, habéis sido grandes maestros.

Gracias también a todo el reparto de Biotecnologie del IZSLT en especial a Antonella Cersini que me ha hecho descubrir un nuevo mundo llamado Biología Molecular muy abstracto y a veces desesperante pero siempre apasionante, a los técnicos Valeria, Silvia, Raffaella y Maurizio, gracias por la ayuda, los trucos y las masterclass de secuencias.

Otro agradecimiento al profesor Vincenzo Veneziano y a los demás componentes del grupo de investigación de la Facultad de Veterinaria de la Universidad Federico II de Nápoles por involucrarme en su estudio y la gran acogida que recibí, recuerdo con mucho cariño esa estancia.

A los propietarios de los animales por su gran disponibilidad y a Mari Cruz Llorente por su inestimable ayuda en los muestreos.

A Ricardo García, de la UCM por la ayuda prestada con la Estadistica.

A la gente de la Facultad: las chicas del 126 Ana, Rocio y Valentina, que siempre me han echado una mano, a Julia y Miguel, que también me han ayudado entre llaves, horarios intempestivos y documentos.

A Marta Hernández, María Alcaide y Daniel Beltrán por la ayuda prestada con las traducciones y las correcciones, y quien que me ha ayudado a resolver imprevistos logísticos, Isaac de Bedoya y Patri Alba.

A mis amigos y residentes en el mundo, todas las experiencias vividas aquí y allí sé que nos unirán estemos donde estemos. Gracias por vuestro apoyo.

Ai regà de Biotecnologie mediche que cambiaron positivamente mi concepto de

italiano medio.

A mi familia, que siempre me ha apoyado en la distancia durante mis andanzas, mis locuras y mis luchas contra molinos de viento.

A Claudio, bambino speciale, grazie di tutto sempre, no hay “rigatoni” suficientes para agradecerte lo que haces por mí.

A mi Layka, la pequeña doctora honoris causa, casi la única constante en este tiempo, mi compañía diurna y nocturana en licenciatura, master y doctorado.

Un gracias final a todo aquel que me animó cuando lo necesitaba durante este recorrido, y también una disculpa a todas las personas a las que no he podido dedicar el tiempo que se merecen en muchos momentos.

INDEX

INDICE

INDICE

INTRODUCCIÓN Y OBJETIVOS

INTRODUCTION & OBJECTIVES

INTRODUZIONE E OBIETTIVI

INTRODUCCIÓN

Los equinos actuales se han desarrollado durante más de 50 millones de años a partir de los *Hyracotherium*, animales de pequeñas dimensiones (30-40 cm a la cruz) con cuatro dedos y dentadura omnívora que habitaban los bosques de Centro y Norteamérica. En este proceso los descendientes se adaptaron a las condiciones de herbívoros habitando prados, su estatura fue aumentando y se modificaron la dentadura y las articulaciones, en especial los dedos hasta quedar solo uno.

El actual género *Equus* apareció en América hace un millón y medio de años, y de ahí, se difundió por Asia, Europa y África. En América se extinguió hace unos 10000 años (última era glaciar); pero en el resto del mundo consiguió adaptarse y sobrevivir. Se cree que el antecesor del caballo actual fuera el tarpán, que se extinguió oficialmente en 1876 en Rusia. El caballo fue reintroducido en América durante la colonización española y en Australia se introdujo en el siglo XVIII.

El caballo fue domesticado hacia el 3000 a.C. en Asia Central, y hacia 1500 a.C ya se usaba en todas las zonas del planeta como medio de transporte. Fue empleado en los conflictos bélicos, aunque este uso decayó en la Edad Moderna, quedando reducido al transporte y carga.

La explotación del equino en España es una de las más antiguas, siendo nuestro país junto a Italia los de mayor censo en Europa y gozando, sobre todo España, de un gran reconocimiento por la conservación genética del caballo de Pura Raza Español. La orientación económica del sector ha cambiado radicalmente: ha pasado de ser un animal de tiro a destinarse al ocio y disciplinas deportivas.

El censo de equinos en España desde el 2007 ha presentado una tendencia hacia el alta desde principio de siglo llegando a superar las 700.000 cabezas en el 2013. Desde hace unos años se ha producido una significativa reducción como consecuencia de la fuerte crisis económica que azota el país en este último decenio y durante la cual se ha incrementado exponencialmente el abandono y el sacrificio de animales. Los propietarios, ganaderos y tratantes no podían hacer frente a los gastos ni vender ni alimentar a sus caballos, lo que aumentó el sacrificio de animales o su destino para producción de carne. El uso ilícito de

canales de équidos comercializadas como de otras especies causó una gran alarma social, tanto por el fraude del etiquetado, como por el posible uso de los animales no criados para carne que hubieran podido recibir tratamientos con productos (por ejemplo, flunixin meglumina) con periodos de espera largos, y quizás no respetados.

El sector español de cebo en équidos cría fundamentalmente animales para exportación a países como Francia e Italia ya que el consumo de este tipo de carne en territorio español no se ha incrementado y se reduce a zonas de la mitad norte del país, Cataluña y Comunidad Valenciana.

Los datos ministeriales determinan en 2016 un censo de caballos de razas puras registrados de 341.963 cabezas. Las razas presentes en nuestro país se dividen en diferentes secciones: razas integradas (angloárabe, árabe pura sangre inglés, trotador español), razas autóctonas en fomento (española), razas autóctonas en peligro de extinción (asturcón, burguete, caballo de monte del País Vasco, caballo de pura raza gallega, cavall pirinenc català, hispano-árabe, hispano-bretón, jaca navarra, losina, mallorquina, marismeña, menorquina, monchina, pottoka) y otros équidos (caballo de deporte español). En el sector asnal, hay censados 3.892 animales y todos ellos son de razas autóctonas en peligro de extinción (andaluz, asno de las Encartaciones, balear, zamorano-leonés y catalán).

En el 2008 se establecieron las ordenanzas en materia de zootecnia creando el Programa Nacional de Conservación, Mejora y Fomento de las Razas Ganaderas (Real Decreto 2129/2008), si bien ya se habían llevado a cabo programas desde 1993. Estos programas buscan la conservación, mejora y/o fomento de las razas y en función del censo, catalogación y características, pueden ser tanto de selección como de conservación. Actualmente es competencia de las asociaciones reconocidas y registradas desarrollar el programa de mejora aprobado por la autoridad competente.

En Italia también se ha evidenciado un cambio de orientación en el sector, pasando igualmente de un contexto bélico y agrícola al ocio y deporte, pero a diferencia de España es el primer productor de carne de caballo en Europa. Varias entidades como la Associazione italiana allevatori (Aia) y la Unione nazionale incremento razze equine (Unire) han fomentado programas de protección y desarrollo de las razas con la intermediación del Ministerio de

Agricultura. Actualmente hay 475.435 équidos registrados a nivel nacional. Las principales razas de caballos incluyen cavallo agricolo italiano da tiro pesante rapido, anglo-árabe, árabe, avelignese, bardagiano, lipizzano, murgese, silla italiano, caballo oriental, pura sangre inglés, pura sangre siciliano oriental y trotador. Existen otras razas que entran en la categoría llamada de "baja difusión" como son los caballos appenninico, della Giara, del Catria, del Delta, maremmano, di Monterufoli, napolitano, persano, salernitano, sarcidano, sanfratellano, tolfetano, del Ventasso, del Pentro y el pony Esperia. En esta categoría se engloban también todas las razas de burros: amaiata, asino dell'asinara, Martina Franca, ragusano, romagnolo, pantesco, sardo y viterbese.

En cuanto a la situación zoosanitaria de España e Italia en relación a las enfermedades equinas de la lista de la OIE, durante los últimos diez años se han observado focos en áreas limitadas de infección por el herpesvirus equino 1 (HVE-1), virus de la arteritis equina y virus West Nile de la encefalomielitis equina en el 2008 en Italia y 2010 en España. En España se han registrado casos de gripe equina y de metritis contagiosa que no se observaban en Italia desde el 1999 y 2010 respectivamente. En Italia se han detectado focos de anemia infecciosa (presentes en todo el decenio) y casos de durina (2011) que no se registraban en España desde el 1983 y 1955. En ambos países no se han observado casos de muermo, peste equina (erradicada en España en 1990) ni de encefalomielitis equina venezolana (nunca descrita). La piroplasmosis equina es enzoótica en las dos Penínsulas.

La piroplasmosis equina es una enfermedad que afecta a los équidos causada por protozoos de los géneros *Theileria equi* y *Babesia caballi* que se transmite a través de vectores ixódidos. Se puede presentar en diferentes formas: hiperaguda, aguda, crónica, siendo la más frecuente la forma aguda. Los signos clínicos más característicos son fiebre, anemia e ictericia. Las técnicas de diagnóstico que recomienda la OIE son las técnicas inmunológicas (ELISA e IFI) si bien se pueden evidenciar los parásitos mediante frotis sanguíneo y métodos moleculares.

Esta enfermedad genera grandes pérdidas económicas, no solo originadas por el tratamiento y disminución del rendimiento de los animales que la padecen, sino por las restricciones en la importación de animales portadores en muchos países.

En Italia, el primer caso de piroplasmosis equina lo describe Guglielmi en 1899 en

Apulia, como un caso de malaria del caballo, en el texto describe las características del frotis que realiza e hipotetiza sobre el vector de transmisión. El primer caso clínico de piroplasmosis en España fue descrito por Almarza en Extremadura en 1933, quien en su publicación describe ampliamente las características y las diferencias entre *Piroplasma caballi* y *Nuttalia equi* y hace hincapié en la necesidad de mayores estudios sobre la enfermedad y los parásitos que la causan pues lo considera una patología frecuente.

Con el paso de los años y la continua presencia en el mismo nicho de hospedadores, vectores y parásitos, sigue siendo una enfermedad frecuente que conlleva importantes pérdidas y requiere de continuos estudios para conocer la situación.

OBJETIVOS

- Confirmar la situación epidemiológica en zonas endémicas de piroplasmosis para una mejor interpretación de las distintas técnicas de detección y diagnóstico de la enfermedad; determinando la prevalencia y evaluando las diferentes técnicas disponibles, poniendo a punto protocolos de diagnóstico molecular.

- Verificar los factores extrínsecos e intrínsecos relacionados con la aparición de casos clínicos, evaluando potenciales factores de riesgo asociados y caracterizando los genotipos circulantes determinando su patogenicidad y distribución.

- Comparar la presentación de casos clínicos en dos áreas endémicas y establecer protocolos de diagnóstico.

EN

INTRODUCTION

Today's equines have developed for more than 50 million years from the *Hyracotherium*, small animals (30-40 cm to the withers) with four fingers and omnivorous teeth that lived in Central and North American forests. During the evolutionary process their descendants adapted to the condition of herbivorous living in pastures, the highness increased, the teeth changed as well as extremities, changing the fingers to one. The current *Equus* gender appeared in America 1.5 million years ago, then extended to Asia, Europe and Africa. In America, it extinguished around 10000 years ago (last ice age); but it managed to adapt and survive in the rest of the world. It is thought that the ancestor of the present horse was the tarpan that officially extinguished in 1876 in Russia. Horses were reintroduced in America during the Spanish colonization and it was introduced in Australia in the XVIII century.

Horses were domesticated around 3000 b.C. in Central Asia, and around 1500 b.C. it was already used in the entire world as a means of transport. It was used in wars,

IT

INTRODUZIONE

Gli equini attuali si sono sviluppati per oltre 50 milioni di anni dai *Hyracotherium*, animali di piccola taglia (30-40 cm al garrese) con quattro dita e dentizione da onnivoro che abitavano nelle foreste di centro e nord America e in questo processo, i loro discendenti si adeguarono alle condizioni di erbivori abitando prati, aumentando l'altezza, modificando le articolazioni, la dentizione e anche le dita fino a restarne solo uno. L'attuale genere *Equus* comparse in America un milione e mezzo di anni fa, e da lì si diffuse in tutta l'Asia, Europa e Africa. In America si estinse circa 10.000 anni fa (l'ultima era glaciale); ma nel resto del mondo riuscì ad adattarsi e sopravvivere. Si ritiene che il predecessore del cavallo attuale fosse il Tarpan, che si estinse ufficialmente nel 1876 in Russia. Il cavallo fu reintrodotto in America durante la colonizzazione spagnola e in Australia nel XVIII secolo.

Il cavallo fu addomesticato circa nel 3000 a.C. in Asia centrale, e verso il 1500 a.C. era già utilizzato in tutte le aree del pianeta come un mezzo di trasporto. È stato impiegato nei conflitti armati, anche se

although its use decayed during the Modern Age, being reduced to transport and cargo.

Equine breeding in Spain is one of the oldest, having our country, together with Italy, the higher census in Europe and having, specially Spain, an important recognition from the genetic conservation of the Spanish purebred horse.

Since 2007, the Spanish equine census has presented an uptrend, reaching over 700000 animals in 2013. However, the last few years a significant reduction has occurred due to the important economic crisis that the country has suffered in this last decade. During this time the abandonment and slaughter of animals has increased exponentially by the owners, farmers and dealers that could not cope with costs neither feed their horses thus, incrementing animal bait and meat production sectors. The illicit use of equidae meat commercialised as from other species caused a great social alarm because of labeling fraud and for the possible use of animals not destined to human consumption which could have received drug treatments (eg. flunixin meglumine) with long suppression periods.

questo uso diminuì nell'Età Moderna, riducendosi al trasporto e al carico.

Lo sfruttamento dei cavalli in Spagna è uno dei più antichi, il nostro paese insieme all'Italia sono i paesi di maggior censimento in Europa e soprattutto la Spagna gode di un grande riconoscimento per la conservazione genetica del Razza Pura Spagnola. L'orientamento economico del settore è cambiato radicalmente, da un animale da tiro, si è passato ad un animale destinato al tempo libero e alle discipline sportive.

Dal 2007 il censimento di equini in Spagna, verifica una tendenza all'aumento superando i 700.000 capi nel 2013, anche se da alcuni anni si è ridotto significativamente a causa della grave crisi economica che affligge il paese in questo decennio e nella quale è aumentato in modo esponenziale l'abbandono e la macellazione di animali da parte di proprietari, allevatori e commercianti che non hanno potuto affrontare le spese di sostentamento, né vendere i capi, né nutrirli, aumentando così il settore d'ingrasso degli animali e la produzione di carne. L'uso illecito di carne equina commercializzata come di altre specie, ha

Bait sector in Spain is aimed to export to countries as France or Italy, since consumption of this kind of meat in the Spanish territory is basically reduced to mid-north areas of the country such as Cataluña and the Comunidad Valenciana.

Ministerial data establishes a census of purebred registered horses of 341963 heads (2016). Breeds present in our country are divided into different sections: integrated breeds (Anglo-Arab, English Purebred Arabian, Spanish Trotter), native breeds in development (Spanish), native breeds in danger of extinction (Asturcón, Burguete, Horse of the Basque Country, Horse of Pure Galician Breed, Cavall pirinenc Català, Hispano-Arabic, Hispano-Breton, Jaca navarra, Losina, Mallorquina, Marismeña, Menorquina, Monchina, Pottoka) and other equines (Spanish Sport Horse). As for the donekys, 3892 animals have been registered. All are indigenous breeds in danger of extinction (Andalusian, las Encartaciones donkey, Balearic, Zamorano-Leonese and Catalan). Since 2008, animal husbandry ordinances have been updated, establishing the National Program for the Conservation, Improvement and Promotion of Livestock

causato grande allarme sia per la frode dell'etichettatura sia per il possibile uso degli animali non destinati a consumo che avrebbero potuto ricevere trattamenti con prodotti (ad es. flunixin meglumine) con periodi di soppressione lunghi. Il settore dell'ingrasso alleva animali fondamentalmente per l'esportazione a paesi come la Francia e l'Italia, dovuto al fatto che il consumo di questo tipo di carne in territorio spagnolo non è aumentato e si riduce soltanto alla metá settentrionale del paese, nella Catalogna e Comunità di Valencia. I dati ministeriali del 2016 determinano un censimento di cavalli di razze pure registrati di 341.963 capi. Le razze presenti nel nostro paese sono divise in diverse sezioni: razze integrate (Anglo-araba, Arabo, Purosangue Inglese, Trottatore Spagnolo), razze autoctone in promozione (Spagnolo), razze autoctone in via di estinzione (Asturcón, Burguete, Cavallo di Montagna del Paese Basco, Cavallo di Razza Pura Galego, Cavall Pirinenc Català, Ispano-arabo, Ispano-bretone, Jaca Navarra, Losina, Mallorquina, Marismeña, Menorquina, Monchina, Pottoka) e altri equini (Cavallo da Sport Spagnolo). Nel settore asinino, sono stati registrati 3.892 animali, tutti di razze

Breeds (Royal Decree 2129/2008), although programs have been carried out since 1993. These programs seek conservation, and/or promotion of breeds and, according to the census, cataloging and characteristics, can be of selection or conservation. It is the competence of recognized associations to develop the improvement program approved by the competent authority.

In Italy, there has also been a change in orientation in the sector, from a military and agricultural context to leisure and sport, although it is the leading producer of horse meat in Europe. Several organizations such as the Associazione italiana allevatori (Aia) and the Unione nazionale increase razze equine (Unire) have promoted programs for the protection and development of breeds with the intermediary of the Ministry of Agriculture. There are currently 475435 registered equidae in the national registry; The main breeds of horses include Italian heavy draft, Anglo-Arabian, Arabian, Avelignese, Bardagiano, Lipizzan, Murgese, Italian saddle, Oriental horse, English thoroughbred, trotter and Eastern Sicilian thoroughbred. There are other breeds under a category called “low diffusion” like

autoctone minacciate di estinzione (Asino Andaluso, “Asino di las Encartaciones”, Balear, Zamorano-leonés, Catalano). Nel 2008 si stabilì il primo Programma Nazionale di conservazione, miglioramento, e promozione delle razze da allevamento, regolato dal Regio Decreto n. 2129/2008, anche se già dal 1993 erano in corso alcuni programmi. L’obiettivo di questi programmi è la conservazione, il miglioramento e/o lo sviluppo delle razze, e secondo il loro censimento, catalogazione e caratteristiche, possono essere di selezione o conservazione. Spetta alle associazioni riconosciute di sviluppare il programma di miglioramento approvato dall'autorità competente.

Sebbene l’Italia è il più grande produttore di carne equina dell’ Europa, si è anche evidenziato un riorientamento del settore equino, passando da un contesto militare ed agricolo ad un altro dedicato al tempo libero e allo sport. Diverse organizzazioni, come l’Associazione Italiana Allevatori (Aia) e Unione nazionale incremento razze equine (Unire), hanno promosso programmi di protezione e sviluppo delle razze con l’intermediazione del Ministero dell'Agricoltura. Attualmente nel Registro Nazionale sono registrati

Appenninico, della Giara, Catria, Delta, Maremmano, di Monterufoli, Neapolitan, Persano, Salernitano, Sarcidano, Sanfratellano, Tolfetano, Ventasso, Pentro and pony Esperia. This category also includes all races of donkeys: Amaiata, Asino dell'asinara, Martina Franca, Ragusano, Romagnolo, Pantesco, Sardo and Viterbese.

Regarding animal health status in Spain and Italy in relation to equine diseases listed by the OIE, several foci have been observed in the last 10 years in limited areas of infection with equine herpesvirus 1 (HVE-1), equine arteritis and West Nile virus from equine encephalomyelitis in 2008 in Italy and 2010 in Spain. In Spain there have been cases of equine influenza and contagious metritis that were not observed in Italy since 1999 and 2010 respectively. In Italy, outbreaks of infectious anemia (present throughout the period) and cases of dourine (2011), which have not been recorded in Spain since 1983 and 1955, have been detected. In both countries there have been no cases of glanders, African horse sickness (eradicated in Spain in 1990) or Venezuelan equine encephalomyelitis (never described).

475.435 equini; le principali razze equine includono: Cavallo Agricolo Italiano da Tiro Pesante Rapido, Anglo-arabo, Arabo, Avelignese, Bardagiano, Lipizzano, Murgese, Sella Italiano, Cavallo Orientale, Purosangue Inglese, Puro Sangue Siciliano Orientale e Trottatore, esistono in piú altre razze categorizzate come "a bassa diffusione", tali come il Cavallo Appenninico, della Giara, del Catria, del Delta, Maremmano, di Monterufoli, Napoletano, Persano, Salernitano, Sarcidano, Sanfratellano, Tolfetano, del Ventasso, del Pentro e il pony Esperia. Questa categoria comprende anche tutte le razze di asini: amiata, asino dell'Asinara, Martina Franca, Ragusano, Romagnolo, Pantesco, Sardo e Viterbese.

Per quanto riguarda la situazione zoosanitaria in Spagna e in Italia, in relazione alle malattie equine inserite nell'elenco dell'OIE, nel corso degli ultimi dieci anni, si sono riscontrati focolai in zone circoscritte d'infezione da herpesvirus equino 1 (HVE- 1), virus della arterite equina, virus del West Nile, virus della encefalomielite equina nel 2008 in italia e nel 2010 in Spagna. In Spagna si sono registrati casi di influenza equina e di metrite contagiosa che non erano stati

Equine piroplasmosis is enzootic in both peninsulas.

Equine piroplasmosis is a disease affecting equidae caused by protozoa of the genera *Theileria equi* and *Babesia caballi.* It is transmitted through ixodids vectors. It can develop in different forms: hyperacute, acute, and chronic. The most frequent form is the acute. The most characteristic clinical signs are fever, anemia and jaundice. The diagnostic techniques recommended by the OIE are ELISA and IFAT although parasites can also be evidenced by blood smear and molecular methods. This disease generates economic losses, not only caused by treatment costs and reduction of the animal performance, but also by the restrictions on the importation of animal carriers in many countries.

In Italy, the first case of equine piroplasmosis is described by Guglielmi in 1899 in Apulia, as a case of horse malaria. It describes the characteristics of the blood smear and hypothesizes about the transmission vector. The first clinical case of piroplasmosis in Spain was described by Almarza in Extremadura, in 1933. In his publication, he extensively describes the characteristics and differences between

osservati in Italia dal 1999 e dal 2010 rispettivamente. In Italia, sono stati rilevati focolai di anemia infettiva (presenti lungo tutto il periodo) e di durina (2011) che non sono stati registrati in Spagna dal 1983 e 1955. In entrambi i paesi non sono stati segnalati casi della morva, peste equina (eradicata in Spagna nel 1990) e di encefalomielite equina venezuelana (mai descritta). La piroplasmosi equina è enzootica nelle due penisole.

La piroplasmosi equina è una malattia che colpisce equidi causata da protozoi del genere *Theileria equi* e *Babesia caballi* trasmessa attraverso vettori del genere Ixodidae. Può avvenire in diversi modi: iperacuto, acuto (il più frequente), e cronico. I segni clinici più caratteristici sono febbre, anemia e ittero. Le tecniche diagnostiche raccomandate dall'OIE sono IFI ed ELISA, anche se i parassiti possono essere evidenziati con striscio sanguigno e metodi molecolari. Questa malattia non solo genera perdite economiche causate dal trattamento e dal calo del rendimento degli animali che la soffrono, ma anche restrizioni all'importazione di animali portatori in molti paesi. In Italia, il primo caso di piroplasmosi equina fu descritto da Guglielmi nel 1899 in Puglia come un caso

Piroplasma caballi and *Nuttalia equi*, and emphasizes the need for further studies on the disease and parasites since it is a frequent pathology.

Throughout the years and the continuous presence in the same niche of hosts, vectors and parasites, equine piroplamosis is still a frequent disease that entails important losses and requires continuous studies to know the situation.

di malaria del cavallo. Nel testo, Guglielmi descrive le caratteristiche di strisci effettuati e fa ipotesi sulla trasmissione vettoriale. Il primo caso clinico di piroplasmosi in Spagna è stato descritto da Almarza in Estremadura nel 1933. Almarza nella sua pubblicazione descrive in profondità le caratteristiche e le differenze fra *Piroplasma caballi* e *Nuttalia equi* e sottolinea la necessità di ulteriori studi sulla malattia e sui parassiti causanti, risaltando che è una patologia frequente.

Nel corso degli anni e la presenza continua nella stessa nicchia di ospiti, vettori e parassiti, la piroplasmosi equina rimane una malattia comune che porta perdite significative e richiede continui studi per capire la situazione.

EN

OBJECTIVES

-To confirm the epidemiological situation in endemic areas of piroplasmosis for a better interpretation of the different detection techniques and diagnosis of the disease. Determing the prevalence and evaluating the different techniques available setting up new molecular diagnostic protocols.

IT

OBIETTIVI

-Confermare la situazione epidemiologica in aree endemiche di piroplasmosi per una migliore interpretazione delle diverse tecniche di rilevamento e diagnostica della malattia. Determinando la prevalenza nelle aree ed evaluando le diverse tecniche disponibili, mettendo a punto protocolli di diagnostica

- To verify the extrinsic and intrinsic factors related to the appearance of clinical cases; evaluating potential risk factors associated and characterizing circulating genotypes, determining their pathogenicity and distribution.

-To compare the presentation of clinical cases in two endemic areas and establish diagnostic protocols.

.

molecolare.

-Verificare i fattori estrinseci ed intrinseci legati alla comparsa di casi clinici; studiando potenziali fattori di rischio associati e caratterizzando i genotipi circolanti, determinandone la patogenicità e distribuzione.

-Paragonare la presentazione di casi clinici in due aree endemiche e stabilire protocolli diagnostici.

PIROPLASMOSIS EQUINA

EQUINE PIROPLASMOSIS **PIROPLASMOSI EQUINA**

ANTECEDENTES DEL TEMA

Este trabajo se presenta por capítulos independientes que contienen una extensa bibliografía en cada uno de ellos. En este capítulo inicial se realiza una exposición más somera sobre la situación del arte del conocimiento de las piroplasmosis que no incluye todo lo posteriormente revisado.

La piroplasmosis equina es una enfermedad causada por diversos protozoos pertenecientes al Phylum Apicomplexa, Orden Piroplasmida (Pirum = pera en latino por su forma, o pyros= fuego en griego por la fiebre) *Babesia caballi*, Nutall 1910 y *Babesia equi*, Laveran 1901 reclasificada como *Theileria equi* por Mehlhorn y Schein en 1998 utilizando análisis moleculares y confirmando el estadio pre-eritrocitario de su ciclo biológico.

A finales del siglo XIX, Babes descubrió microorganismos en los eritrocitos de un ternero en Rumanía y los asoció con la hemoglobinuria bovina o fiebre del agua roja (Babes, 1888). El género *Theileria* fue descrito por primera vez por Koch en 1898. En 1905 Koch postuló que la fiebre biliar de los caballos estaba causada por dos patógenos, ahora conocidos como *Babesia caballi* y *Theileria equi.* Nuttall y Strickland en 1910 hicieron una clara distinción de la forma intraeritrocitaria de ambos microorganismos durante sus observaciones.

Los piroplasmas de *Babesia* son ameboides, circulares u ovales con una longitud de 2 a 5 µm. *Theileria* presenta una forma oval, circular, anular o piriforme y mide de 1,5 a 2,5 µm disponiéndose en forma de cruz de Malta.

Las especies hospedadoras de estos agentes son caballos, asnos, mulos y cebras. Los parásitos en el hospedador vertebrado se encuentran en los glóbulos rojos y blancos. Los animales infectados pueden seguir siendo portadores durante varios años en el caso de *B.caballi* (De Waal y van Heerden, 1994) o durante toda la vida del animal en el caso de *T.equi* (Brüning, 1996). Los animales nacidos en zonas endémicas pueden actuar como portadores sanos (Sippel y col., 1962). La terapia farmacológica en infecciones causadas por *T. equi* no consigue eliminar el parásito completamente (De Waal, 1992) y puede ocurrir que en situaciones de estrés, tratamiento inmunodepresivo o enfermedad concomitante los piroplasmas se reactiven, multiplicándose y reapareciendo en sangre periférica.

De los 120 millones de caballos que hay en el mundo, el 90% viven en zonas endémicas; además de los gastos producidos por la mortalidad y coste de los tratamientos, es importante la repercusión de esta enfermedad que disminuye el rendimiento deportivo de los animales (Hailat y col., 1997) y limita el movimiento de los mismos a concursos, competiciones internacionales y Olimpiadas, así como en general las restricciones de los animales positivos que afectan negativamente al comercio internacional de équidos.

Los parásitos son transmitidos principalmente por garrapatas duras de la familia Ixodidae (ninfas y adultas), aunque se han observado otra vías de transmisión como la mecánica, a través de vectores contaminados con sangre infectada (por ejemplo agujas), la venérea, en el caso en que la sangre entre en contacto con el semen (Metcalf, 2001) y la transmisión transplacentaria que no se había determinado en *B. caballi,* pero sí en el caso de *T.equi* (Georges y col., 2011).

Las garrapatas en los équidos se localizan en las orejas, divertículo nasal y en casos de grande infestación en la frente, cabeza, cuello, pecho y región perineal (Pfeifer Barbosa y col., 1995). Se han identificado de 12 a 14 especies de garrapatas transmisoras, siendo los géneros principales *Anocentor, Dermacentor, Rhipicephalus, Hyalomma, Boophilus, Ixodes* y *Haemophysalis* (Scoles y Ueti, 2015). En Europa, *B.caballi* se transmite principalmente por garrapatas del género *Dermacentor* y *T.equi* por garrapatas de los géneros *Rhipicephalus* y *Hyalomma;* las especies presentes en Italia y España son *Dermacentor marginatus, Rhipicephalus bursa, Riphicephalus turanicus* e *Hyalomma marginatum;* en España además, está presente *Hyalomma lusitanicum* (Habela y col., 1989; Iori y col., 2010).

Babesia caballi se encuentra en diferentes órganos de la garrapata y pueden actuar como vectores transestadiales; además, 8 de estas especies son también capaces de transmitir *B.caballi* de forma transovárica. *Theileria equi* se desarrolla en las glándulas salivares, saliva, intestino y hemolinfa de la garrapata (Mehlhorn y Schein, 1998), observándose alteraciones estructurales en las glándulas salivares de garrapatas infectadas con *T.equi* causadas por el parásito (Kumar y col. 2007). A diferencia de *B.caballi* en *T.equi* no hay constancia de paso transovárico, solo transestadial.

Cuando las garrapatas infectadas se alimentan del équido hospedador, le inoculan

esporozoítos; los esporozoítos de *B. caballi* invaden los eritrocitos y se transforman en trofozoítos que crecen dividiéndose cada uno en dos merozoítos de forma oval o piriforme, que son capaces de infectar otros glóbulos rojos repitiéndose el proceso de división.

En el caso de *T. equi* los esporozoítos inoculados invaden los linfocitos y estas formas intraleucocitarias se desarrollan formando esquizontes, éstos liberaran merozoítos que invaden los eritrocitos transformándose en trofozoítos que crecen y se dividen en merozoítos infectantes con forma de pera que se disponen en tétradas (cruz de Malta) en el glóbulo rojo.

Las diferencias entre *Babesia* y *Theileria* basadas en el ciclo biológico (presencia de una fase pre-eritrocitaria en *T. equi*), los vectores que las transmiten y la modalidad (transestadial y transovárica o solo transestadial), así como las diferencias morfológicas y la diferente susceptibilidad a los fármacos usados para el tratamiento hicieron que Mehlhorn y Schein en 1998 reclasificaran *Babesia equi* como *Theileria equi*.

Se ha observado una discreta especificidad entre parásito y especie hospedadora causada a su vez por la baja especificidad del vector (Navarrete y col., 1999) lo que puede determinar la presencia en una especie animal de piroplasmas distintos a los propios de la especie. Así pues se han aislado en caballos otros parásitos como *Encephalitozoon* (Ribeiro y col., 2006), *Babesia canis canis* (Criado-Fornelio y col., 2003; Fritz, 2010; Zanet y col., 2017), *Babesia canis rossi* (Fritz, 2010), *Babesia bovis* en animales sintomáticos (Criado y col., 2006), *Theileria annae, Theileria sergenti* y *Theileria buffeli* (Moretti y col., 2010). Al contrario también se han encontrado piroplasmas equinos en otras especies como *Theileria equi* o *Babesia caballi* en perros y dromedarios (Criado-Fornelio y col., 2003; Beck y col., 2009; Fritz, 2010; Qablan y col., 2012)

Se han descubierto más de 30 especies de piroplasmas pertenecientes a los géneros *Theileria* y *Babesia* (Criado Fornelio y col., 2004). Según Criado-Fornelio y col. (2003), la evolución de los Piroplásmidos de los mamíferos placentarios podría haber sucedido en África hace de 55 a 20 millones de años y *T.equi* podría ser el ancestro de los Theiléridos actuales debido a sus características primitivas. En su estudio describen a *T.equi* africana como ancestro de las variedades españolas, e indica que *B. caballi* o sus ancestros serían el origen de un linaje de ungulibabésidos del caballo, habiendo pasado el grupo a los bóvidos primitivos y de ahí a

los cápridos.

Para estudiar los diferentes genotipos se han utilizado técnicas de amplificación de ADN, como PCR, RLB y secuenciación a partir de distintos moldes. Las primeras caracterizaciones genotípicas fueron realizadas en España por Nagore y col., (2004), usando la técnica de RLB y utilizando como target el 18S con el que identificó formas nuevas que denominaron "like" además de las clásicas en ambos géneros.

La subunidad del 18S rRNA se ha usado en numerosos estudios porque presenta regiones conservadas y a la vez variables lo que permite alineamientos inequívocos y una posterior discriminación filogenética; otras subunidades han sido poco utilizadas, como la 28S por ejemplo, ya que es demasiado larga y poco relevante para su uso filogenético, mientras las subunidades 5S y 8S se han descartado por ser demasiado cortas.

Además de las secuencias ribosomales se han usado secuencias diana del antígeno del merozoíto (EMA-1) de *T.equi* (Bhoora y col., 2010; Munkhjargal y col., 2013; Ketter-Ratzon y col., 2017), y del gen de la proteína asociada afl rhoptry 1 (RAP-1) de *B.caballi* (Bhoora y col., 2010; Rapoport y col., 2014) que han mostrado una gran heterogeneidad, si bien la variabilidad presente en *B.caballi* parece inferior debido probablemente al menor número de muestras positivas a este parásito encontradas, y a que los bajos niveles de parasitemia dificultan la clasificación genotípica. Otras menos utilizadas han sido el gen de la β-tubulina empleada para la discriminación entre especies (Cacciò y col., 2000) y los genes del citocromo b (Criado y col., 2006).

En los estudios en los que se ha usado el 18S rRNA, EMA y RAP se han identificado clados diferenciados presentes en cada género, con el 18S se han identificado de 3 a 5 grupos para *T.equi* y dos clados (uno con dos subgrupos) para *B.caballi;* las formas clásicas y "like" descritas por Nagore y col. (2004) aparecen en clados diferentes. Usando el antígeno EMA se han evidenciado tres clados y con el RAP dos (uno presenta dos subclados). Las secuencias de las mismas muestras analizadas usando el 18S y un target específico de especie no muestran una exacta correspondencia entre clados identificados con los distintos métodos. Estos estudios se han desarrollado en diferentes países, y no evidencian influencia geográfica en la presencia de los diferentes genotipos ya que la presencia de estos subgrupos se mantiene. No

se han realizado estudios sobre la patogenicidad ni identificado genotipos más virulentos.

La distribución de estas enfermedades está ligada a la presencia del vector por lo que es cosmopolita. Aunque se han descrito casos clínicos de piroplasmosis en todos los continentes, la enfermedad es endémica en las áreas tropicales, subtropicales y en zonas templadas (Brüning, 1996). No se consideran zonas endémicas Islandia, Inglaterra e Irlanda, Australia, Japón, Rusia, Canadá y Estados Unidos (excepto Florida y Tejas). En Australia se introdujo a mediados de los años 70 por la importación de caballos españoles positivos pero la enfermedad no se estableció en la población autóctona (Mahoney y col., 1977), en el sur de Estados Unidos se introdujo con caballos cubanos importados en 1959 y el vector *Dermacentor nitens* la transmitió a caballos nativos. En algunas zonas teóricamente indemnes se han evidenciado animales seropositivos y se sospecha que los vectores hayan sido transportados por animales salvajes o aves migratorias (Butler y col., 2012).

En la cuenca mediterránea las infecciones causadas por *T. equi* son más frecuentes que las producidas por *B. caballi* (Bashiruddin y col., 1999; Criado-Fornelio y col., 2003), sin embargo en África *B. caballi* es más prevalente.

En la siguiente tabla se indica la prevalencia de algunos estudios desarrollados en la cuenca mediterránea en el siglo pasado y presente.

En zonas endémicas, los potros reciben anticuerpos maternos con el calostro (De Waal y Van Heerden, 1994), que pueden mantenerse hasta los cinco o seis meses de edad y que les confieren protección durante este periodo (de Waal y van Heerden, 2004). En un estudio realizado en España por Coleto en 1999, el 90% de los potros nacidos de yeguas positivas se mantenían seropositivos en IFI durante 90-100 días. Es por ello que los potros nacidos en áreas endémicas solo suelen presentar infecciones subclínicas, ya que la inmunidad materna se sustituye gradualmente por una inmunidad activa y estable debido a la continua presencia del parásito (Phipps, 1996).

En los animales que nunca han estado en contacto con el parásito y se infectan, la aparición de anticuerpos se produce aproximadamente diez días después de la infección y en el caso de *B. caballi* los anticuerpos pueden detectarse durante unos cuatro años, y en *T. equi* toda la vida del animal (de Waal, 1992).

Los antígenos inmunodominantes de la superficie de los merozoítos son una diana importante para la respuesta inmune protectora, y por ello, muchos tests ELISA y ensayos de vacunas han usado epítopos de estas proteínas. Estos son las proteínas RAP-1 en *Babesia* y EMA en *T.equi.* Dos proteínas, EMA-1 y EMA-2 no se expresan durante los estadios de desarrollo eritrocitario asexual, sino que estos dos antígenos se expresan solo durante la primera fase de desarrollo sexual (Kumar y col., 2004).

El periodo de incubación de *Theileria equi* en los equinos es de 12 a 19 días (Mehlhorn y Schein, 1998) hasta que los merozoítos se observan en frotis sanguíneo, sin embargo los esquizontes de los linfocitos se pueden observar dos días después de la infección. En la theileriosis, la parasitemia puede ser elevada llegándose a observar hasta un 80% de eritrocitos infectados en animales esplenectomizados (De Waal y van Heerden, 2004). En *B.caballi* el periodo de incubación va de 10 a 30 días, la parasitemia es mucho menor que la observada en casos de theileriosis y se encuentra entorno al 1%. En animales inmunodeprimidos, los tiempos de incubación y prepatencia se acortan, y la severidad de la enfermedad aumenta (Wise y col., 2014).

La piroplasmosis equina es un síndrome febril hemolítico; la fiebre coincide con la lisis eritrocitaria y la liberación repentina de hemoglobina y otros productos. La hemólisis es el resultado del daño mecánico de los eritrocitos debido a la fisión binaria intraeritrocitaria de los trofozoítos, del daño tóxico causado por factores hemolíticos producidos por el parásito e inmunomediado por autoanticuerpos directos contra los componentes de las membranas de los glóbulos rojos infectados y no infectados y de la toxicidad de los factores hemolíticos que libera el piroplasma (Mahmoud y col., 2016). La acción patógena de estos parásitos también se basa en la liberación de sustancias farmacológicamente activas (esterasas) que activan la calicreína, una amina biógena que produce alteraciones circulatorias, vasodilatación, incremento de la permeabilidad vascular, éstasis circulatorio, shock y muerte. Este fenómeno se observa en las babesiosis, en el caso de *T.equi* el parásito actúa primero sobre el sistema mononuclear fagocitario (fase linfoproliferativa) y después ocasiona anemia severa por la elevada parasitemia (Ambawat y col., 1999). Se ha determinado el papel de los antígenos EMA de *T. equi* y de los ácidos siálicos del hospedador en la unión del parásito con el eritrocito observando que eliminando o bloqueando los ácidos siálicos de la membrana eritrocitaria en

el caso de *B.caballi* un menor número de parásitos consiguen invadir la célula, encontrando más merozoítos fuera de los eritrocitos por lo que se reduce la multiplicación de las babesias. En los mismos experimentos con *T.equi* el desarrollo intracelular de los parásitos se ve alterado (Okamura y col., 2005); Hanafusa y col., 1998 estudiaron el papel de algunas citoquinas como el γIFN, αTNF e IL-2 (relacionado con la pirexia), así como el óxido nítrico, indicador de daño tisular del endotelio y edema. Estas citoquinas suelen actuar para controlar y reducir la parasitemia, pero si se rompe el equilibrio pueden causar daño renal y agravar el shock endotóxico durante la septicemia. TNF y NO pueden ayudar a controlar y reducir la parasitemia pero si se rompe el equilibrio o aumentan excesivamente causan daños severos.

La función del bazo en el control de la piroplasmosis se basa en su actividad fagocítica, en la respuesta humoral contra los antígenos en circulación y en su capacidad para contrarrestar la variación genética.

La piroplasmosis equina presenta las siguientes formas clínicas:

-Hiperaguda: es una forma poco frecuente en la que los animales aparecen moribundos o muertos.

-Aguda: es la forma más común, los animales presentan fiebre alta (coincidiendo con la alta parasitemia), debilidad, falta de apetito y malestar, aumento de la frecuencia cardíaca, mucosas congestivas, anémicas o ictéricas y puede observarse la orina de color oscuro y heces pequeñas y secas.

-Subaguda: es parecida a la aguda pero acompañada de pérdida de peso y fiebre intermitente; el color de las mucosas va del rosa pálido al rosa o del amarillo claro al amarillo brillante, se pueden presentar también petequias y equimosis, así como edemas en las partes distales de las extremidades. La motilidad intestinal suele verse afectada y observarse en los animales signos de cólico.

-Crónica: en estos casos, los animales presentan signos clínicos inespecíficos como inapetencia, bajo rendimiento y menor condición corporal. Esta forma es muy frecuente en burros, asociada a una baja parasitemia.

Otros signos clínicos observados son depresión, fiebre intermitente, anorexia, rinorrea, sialorrea, cojeras y encefalitis (sobre todo en infecciones causadas por *Babesia);* síntomas

bronconeumónicos, edema en cabeza y párpados, lagrimeo, conjuntivitis, incoordinación y linfonodos aumentados.

Los individuos aparentemente sanos que son portadores pueden recidivar en ciertas condiciones de estrés o inmunodepresión (Wise y col., 2014). Las formas clínicas causadas por *T.equi* son más graves que las causadas por *B.caballi* y la tasa de mortalidad más elevada. *T.equi* además puede causar infecciones "in utero" provocando abortos o potros con piroplasmosis neonatal, algunos potros pueden nacer como portadores sanos; en éstos últimos el sistema inmune no reconoce como extraño al parásito y los anticuerpos adquiridos con el calostro controlan los niveles de parasitemia durante los primeros meses de vida del animal (Georges y col., 2011; Sant y col., 2016).

Las lesiones observadas van ligadas a la hemólisis: la sangre se encuentra diluida o acuosa y las mucosas aparecen pálidas o ictéricas, también se observa a veces sangre de color rojo oscuro en la vejiga. Puede presentarse una ligera hepatomegalia, y en algunos casos degeneración y necrosis coagulativa en el hígado. El bazo presenta esplenomegalia con una coloración muy oscura y friable. Los riñones son los órganos más afectados presentando una coloración más oscura o clara de lo normal con congestión severa, en la zona medular en la que se pueden observar petequias. En el corazón se observan frecuentes hemorragias en el epicardio, miocardio y endocardio, degeneración vacuolar y congestión con una cierta separación de los haces musculares miocárdicos. Los pulmones pueden presentar congestión aguda, edema y trombosis de los vasos pulmonares; las infecciones secundarias pueden producir lesiones pulmonares poco específicas como enfisema o neumonía. Otras lesiones descritas son: edema subcutáneo, congestión visceral y presencia de líquido en pleura pericardio y peritoneo (Hailat y col., 1997; Hanafusa y col., 1998; Kumar y col., 2009).

Las alteraciones de la serie roja más frecuentes son: recuento eritrocitario, hemoglobina y hematócrito bajos, variaciones relativas en otros parámetros (RDW, MCV, MCH y MCHC) que se producen en el curso de anemia microcítica e hipocrómica. También puede alterarse la actividad de los órganos hematopoyéticos liberándose poiquilocitos y macroeritrocitos (Mahmoud y col., 2016; Sumbria y col., 2017). En cuanto a la serie blanca las observaciones de los autores han sido variables, encontrando casos de leucopenia y leucocitosis, neutropenia y neutrofilia, linfopenia o linfocitosis (Al-Saad y col., 2009; Laus y col.,

2015) y monocitosis (Diana y col., 2007). El recuento plaquetario es bajo, consecuencia de la coagulación intravascular diseminada local y sistémica, la destrucción inmunomediada y secuestro de las plaquetas en el bazo (Beard y col., 2013). Otras alteraciones en parámetros de la coagulación como el fibrinógeno plasmático, D-dímero, tiempo de coagulación, tiempo de protrombina, tromboplastina parcial activada han sido descritas por diferentes autores (Mantran y col., 2004; Al-Saad y col., 2009).

Otros hallazgos frecuentes son el aumento de la bilirrubina causado por la hemólisis (Al-Saad y col., 2009); las transaminasas hepáticas elevadas por degeneración centrolobular y necrosis de los hepatocitos (Camacho y col., 2005; Zobba y col., 2008) y la hipoproteinemia con hipoalbuminemia y alteraciones en las globulinas α2 y γ globulinas (Rubino y col., 2006; Barrera y col., 2010).

Se ha observado también la presencia de marcadores de daño cardíaco debido a una posible alteración miocárdica, así como la CK aumentada por daño muscular (Diana y col., 2007)

También se han reportado altos valores de marcadores de estrés oxidativo y peroxidación lipídica como el malondialdehido, así como altos niveles de glutation sérico y bajos niveles de vitamina E (Ambawat y col., 1999; Deger y col., 2009).

Los factores de riesgo asociados a la piroplasmosis equina descritos recientemente por diferentes autores (García-Bocanegra y col., 2012; Bartolomé del Pino y col., 2016; Zanet y col., 2017; Sumbria y col., 2017) señalan la influencia de características individuales de los équidos como especie, edad, género, raza y aptitud, observándose una mayor prevalencia en mulas y asnos, en hembras, en animales adultos y viejos por la persistencia de anticuerpos y en animales de razas autóctonas y cruzadas que puede deberse a diferencias en la susceptibilidad de las mismas a esta enfermedad. Otros factores de riesgo observados en estos estudios están relacionados con las prácticas de manejo; tipo de alojamiento y acceso al pasto que determinan un mayor contacto con los vectores y la administración de tratamientos repelentes, antiparasitarios internos y vacunación regular definidos como factores protectores al ser indicadores de buenas prácticas. Asimismo se han observado distintos factores relacionados con la ecología del vector como son: el área geográfica, la altura, el clima, el tipo

de suelo y la cobertura terrestre.

En general, estos factores hacen referencia a condiciones que favorecen la presencia de los vectores y un mayor contacto de los animales con los mismos.

En cuanto al diagnóstico cabe señalar que se deben utilizar tanto los métodos clásicos como los más modernos.

-Clínico-epidemiológico, basado en la sintomatología, presencia de garrapatas, focos de la enfermedad cercanos, etc. Este tipo de diagnóstico es poco específico y siempre se debe corroborar con una técnica laboratorial, pero ofrece una información insostituible.

-El diagnóstico diferencial contempla diferentes enfermedades que cursan con una anemia y/o ictericia de origen hemolítico como son la ehrlichiosis, anemia infecciosa equina, peste equina, tripanosomiasis, leptospirosis, intoxicaciones, arteritis, púrpura hemorrágica, etc.

-Parasitológico directo: consiste en evidenciar directamente los piroplasmas a través de frotis o PCR.

-Parasitológico indirecto: se basa en la determinación de anticuerpos, los más representativos son la fijación del complemento, la inmunofluorescencia y el ELISA.

En cuanto a las técnicas parasitológicas directas:

-El frotis sanguíneo se suele hacer extrayendo sangre de capilares superficiales (ollares u orejas) en la fase aguda de la enfermedad (pico febril). La observación directa de piroplasmas también se puede hacer a partir de improntas de órganos durante las necropsias (cerebro, riñón, hígado, pulmones). Estos frotis se tiñen con métodos metacromáticos como Giemsa o Diff-Quick y se observan con el microscopio óptico a inmersión. Algunos límites de esta técnica son la dificultad para identificar los piroplasmas según géneros, la identificación en animales portadores o en casos de baja parasitemia, la necesidad de operadores experimentados y el tiempo de realización para estudios a gran escala.

-La PCR se usa cada vez más para diagnóstico por su elevada sensibilidad y especificidad, de hecho ya existen kits comerciales. Hay muchos estudios experimentales que usan distintas dianas: genes constitutivos u otros antígenos de las formas de replicación como

el merozoíto.

Sobre las técnicas indirectas:

-La fijación del complemento presentaba una gran sensibilidad, y aún se usa para movimientos internacionales en algunos países, pero este test no identifica todos los animales infectados, sobre todo si se han sometido a tratamiento, ya que se producen reacciones anticomplementarias.

-La inmunofluorescencia es una de las pruebas prescritas por la OIE; es un test con mayor sensibilidad que el anterior en casos crónicos, pero presenta reacciones cruzadas entre los dos protozoos (Friedhoff y col., 1986; Brüning y col., 1996), a veces tampoco es fácil diferenciar entre los títulos más bajos y se necesita una gran experiencia interpretativa.

-El ELISA de competición es una prueba ya comercializada que recomienda la OIE. Se usa una proteína recombinante y un anticuerpo monoclonal que define un epítopo de la superficie del merozoíto. El test ELISA es de fácil desarrollo, evita el problema de las reacciones cruzadas, y permite testar muchas muestras a la vez; el problema actualmente es que mientras el test de *T. equi* cubre muchas variedades distribuidas por distintas áreas geográficas, el de *B. caballi* parece que no consigue identificar ciertas variantes de origen africano, pero que también se encuentran en otros lugares (Bhoora y col., 2010; Rapoport y col., 2014). Se han desarrollado experimentalmente otros tipos de ELISA de tipo indirecto que usan proteínas recombinantes, pero no han sido comercializados.

Otros medios diagnósticos encontrados en literatura son el western blot, test de aglutinación en látex y cultivo de sangre in vitro (Holman y col., 1997).

El tratamiento puede tener un objetivo diferente según se trate de países endémicos, donde se centra en salvar la vida del animal y aliviar los síntomas y no se someten a tratamiento los portadores sanos, sin embargo en zonas no endémicas el tratamiento busca, no solo combatir la sintomatología del animal sino eliminar al parásito evitando que el animal quede como portador y propague la infección (Wise y col., 2014).

El tratamiento más frecuente es el dipionato de imidocarb por vía intramuscular. En los burros, se recomienda no pasar la dosis de 2 mg/kg ya que se ha observado que esta especie es más susceptible a los efectos adversos (Singh y col., 1980). Es un tratamiento bastante

eficaz, sobre todo en las infecciones de *Babesia*, las de *Theileria* son más refractarias, habiéndose observado diferencias en la susceptibilidad entre distintas variedades de *T.equi* (Hines y col., 2015), pero tiene indeseables efectos secundarios debido a su actividad anticolinesterásica que puede provocar cólicos, requeriendo siempre una monitorización. Para evitarlos en ocasiones se añade atropina directamente al tratamiento (Abutarbush y col., 2012).

En muchos casos de theileriosis se combina el imidocarb con un antibiótico como la tetraciclina, a la que *T. equi* es sensible en la fase pre-eritrocítica; otras asociaciones son imidocarb y buparvaquone pero actualmente no está aprobado su uso en la CEE (Rothschild 2013).

En cuadros clínicos agudos suele ser necesario un tratamiento de soporte.

Actualmente en muchos países siguen las recomendaciones de la OIE para el movimiento internacional de animales solicitando un certificado donde se indica la ausencia de síntomas el día del embarque, seronegatividad a los test prescritos por la OIE 30 días antes de embarcar y un tratamiento contra ixódidos realizado siete días antes del embarque.

Algunas prácticas útiles para prevenir nuevas infecciones son las medidas de manejo relacionadas con los vectores transmisores por ello se aconseja el uso de antiparasitarios externos (repelentes, acaricidas), limitar las salidas al pasto, inspeccionar a los animales y controlar el ambiente para eliminar posibles garrapatas.

También es fundamental evitar las vías yatrogénicas, evitando compartir agujas entre animales, y usando material desechable.

En el caso de animales sospechosos, se aconseja cuarentena para evitar contagios y proceder al diagnóstico y tratamiento en caso de positividad.

La profilaxis vacunal aún no es posible ya que no se han comercializado vacunas eficaces si bien experimentalmente se probaron vacunas en asnos con esperanzadores resultados (Kumar y col., 2002) pero no se han continuado líneas de investigación para obtenerlas.

Country	*Babesia caballi*		*Theileria equi*	
	XX Century	**XXI Century**	**XX Century**	**XXI Century**
Spain	64.2% IFAT (Habela 1989)	13% IFAT (Olmeda 2000) 21.3% IFAT (Habela 2000) 28.3% IFAT (Camacho 2005) 13.2% cE (G.Bocanegra 2012)	77.1 % IFAT (Habela 1989)	37.6% IFAT (Olmeda 2000) 52.5% IFAT (Habela 2000) 40% IFAT (Camacho 2005) 56.1% cE (G.Bocanegra 2012)
Portugal	15.6% CF (Serra 1993)	11.1% cE (Ribeiro 2013)	45.3% CF (Serra 1993)	17.9% cE (Ribeiro 2013)
France	1.5% CF (Soulé 1998)	12.9% CF (Guidi 2015)	2% CF (Soulé 1998)	58% CF (Guidi 2015)
Italy	20% IFAT (Savini 1997)	56% IFAT (Moretti 2010) 0.3% IFAT (Grandi 2011) 26% IFAT (Laus 2013) 8,9% cE (Bartolomé 2016)	48% IFAT (Savini 1997)	50.48% IFAT (Moretti 2010) 8.2% IFAT (Grandi 2011) 41% IFAT (Laus 2013) 39,8% cE (Bartolomé 2016)
Greece	ND	2.2% cE (Kouam 2010)	ND	10%cE (Kouam 2010)
Turkey	16.5% BS (Inci 1997)	2.3% cE (Sevinc 2008) 19.6% IFI (Karatepe 2009)	9% BS (Inci 1997)	17.67% cE (Sevinc 2008) 12.8% IFI (Karatepe 2009)
Jordan	ND	0% cE (Abutarbush 2012)	ND	14.6% cE (Abutarbush 2012)
Israel	ND	ND	33.7%cE (Shkap 1998)	26.4%cE (Steinman 2012) 43.8cE (Aharonson-Raz 2014)
Egypt	ND	22.3% IFAT (Mahmoud 2016)	ND	26.6% IFAT (Mahmoud 2016)
Tunisia	ND	ND	ND	12.5% PCR (Ros Gª 2013)
Morocco	ND	ND	ND	67% E (Rhalem 2001)

(cE, competitive ELISA; IFAT, indirect fluorescence antibody test; CF, complement fixation)

EN

EQUINE PIROPLASMOSIS

This work is presented by independent chapters that contain an extensive bibliography in each of them. In this initial chapter, a brief exposition is presented about the state of art on piroplasmosis, which does not include all material later revised.

Equine piroplasmosis is a disease caused by several protozoa from the phylum Apycomplexa, Order Piroplasmida (Pirum = pear in latin, due to its shape): *Babesia caballi* (Nutall, 1910) and *Babesia equi* (Laveran, 1901), reclassified as *Theileria equi* by Mehlhorn and Schein in 1998 using molecular analyses and confirming the pre-erythrocyte state of its biological cycle.

At the end of the 19th century, Babes discovered microorganisms in the erythrocytes of a calf in Romania and associated them with bovine hemoglobinuria or red water fever (Babes, 1888). The genus *Theileria* was first described by Koch in 1898. In 1905 Koch postulated that the bile fever of the horses was caused by two pathogens, now known

IT

PIROPLASMOSI EQUINA

Questo lavoro si presenta per capitoli indipendenti, che contengono una estesa bibliografia in ciascuno di loro. In questa parte iniziale si fa un'esposizione generale sullo stato dell'arte della piroplasmosi che non include tutto il materiale posteriormente revisato.

La piroplasmosi equina è una malattia causata da diversi protozoi appartenenti al Phylum Apicomplexa, Ordine Piroplasmida (Pirum = pera in latino per la sua forma) *Babesia caballi* (Nutall, 1910) e *Babesia equi* (Laveran, 1901) riclassificata come *Theileria equi* da Mehlhorn e Schein nel 1998 utilizzando analisi molecolari e confermando lo stadio pre-eritrocitario del suo ciclo biologico.

Alla fine del secolo XIX, Babes descrisse microrganismi negli eritrociti di un vitello in Romania e li associò con l'emoglobinuria bovina o febbre dell'acqua rossa (Babes, 1888). Il genere *Theileria* fu descritto per la prima volta da Koch nel 1898. Nel 1905 Koch postulò che la febbre biliare dei cavalli era causata da due patogeni, ora conosciuti come *Babesia caballi* e *Theileria equi.* Nuttall e Strickland

as *Babesia caballi* and *Theileria equi*. Nuttall and Strickland in 1910 made a clear distinction between the intra-erythrocyte states of both microorganisms during their observations.

Babesia piroplasms are ameboid, circular or oval, with a length of 2 to 5 µm. *Theileria* has an oval, circular, annular or pyriform shape and measures 1.5 to 2.5 µm and is in the form of a Maltese cross.

The host species of these piroplasms are horses, donkeys, mules and zebras. The parasites in the vertebrate host are found in red and white blood cells. Infected animals may continue to be carriers for several years in the case of *B.caballi* (de Waal and van Heerden, 1994) or throughout the life of the animal in the case of *T.equi* (Brüning, 1996). Animals born in endemic areas can act as healthy carriers (Sippel *et al*., 1962). Pharmacological therapy in infections caused by *T.equi* cannot completely eliminate the parasite (De Waal, 1992) and it may happen that in situations of stress, immunosuppressive treatment or concomitant disease the piroplasms are reactivated, multiplying and reappearing in

nel 1910 diedero una chiara distinzione della forma intraeritrocitaria di entrambi i microrganismi durante le loro osservazioni.

Il piroplasma di *Babesia* presenta forma ameboide, circolare e ovale con una longitudine di 2 a 5 µm*. Theileria* presenta una forma ovale, circolare, anulare o piriforme e misura da 1,5 a 2,5 µm disponendosi nella forma della croce di Malta.

Le specie che ospitano questo piroplasma sono cavalli, asini, muli e zebre. I parassiti nell'ospite vertebrato si trovano nei globuli rossi e bianchi. Gli animali infettati possono essere portatori per diversi anni in infezioni sostenute da *B.caballi* (De Waal e van Heerden, 1994) o durante tutta la vita dell'animale nei casi di *T.equi* (Brüning, 1996). Gli animali nati in zone endemiche, possono essere portatori sani (Sippel *et al*., 1962). La terapia farmacologica nell'infezione causata da *T. equi,* non riesce ad eliminare il parassita completamente (de Waal, 1992) e può accadere che in situazioni di stress, trattamento inmunodepressivo o malattia concomitane, il piroplasma si riattivi, moltiplicandosi e riapparendo nel sangue periferico.

peripheral blood.

Of the 120 million horses in the world, 90% live in endemic areas; In addition to the costs caused by mortality and pharmacological treatments, it is also important the repercussion of this disease that diminishes the sports performance of the animals (Hailat *et al.*, 1997) and limits the movement of those to international competitions and Olympics. In general, there are restrictions to positive animals that negatively affect the international trade of equids.

Parasites are mainly transmitted by Ixodid ticks (nymphs and adults), although other transmission routes have been observed such as mechanical, through vectors contaminated with infected blood (i.e. needles), venereal, in the case blood contacts semen (Metcalf, 2001), and transplacental in the case of *T. equi* (Georges *et al.*, 2011). This last route of transmission had not been determined for *B. caballi*.

In equids, ticks are located in the ears and the nasal diverticulum, and in cases of large infestation in the forehead, head, neck, chest and perineal region

Dei 120 millioni di cavalli che vi sono nel mondo, il 90% vive in zone endemiche; oltre alle spese sostenute per la mortalità e per i trattamenti, è importante la ripercussione di questa malattia che diminuisce le prestazioni atletiche di questi animali (Hailat *et al.*, 1997) e limita il movimento di questi ultimi a concorsi, competizioni internazionali e Olimpiadi. In generale, le restrizioni nella movimentazione degli animali positivi,si ripercuotono negativamente sul commercio internazione di equidi.

I parassiti sono trasmessi principalmente tramite zecche Ixodidae (ninfe e adulti) nonché si sono osservate altre vie di trasmissione come la meccanica attraverso vettori contaminati con sangue infetto (per esempio aghi), per via venerea nel caso che il sangue entri in contatto con il liquido seminale (Metcalf, 2001) e la trasmissione transplacentare che non era stata determinata in *B. caballi,* ma determinata nel caso di *T.equi* (Georges *et al.,* 2011).

Le zecche negli equini si localizzano nelle orecchie, nel diverticolo nasale e in caso di grandi infestazioni sulla fronte, testa, collo, petto e regione perianale

(Pfeifer Barbosa *et al*., 1995). Twelve to fourteen species of ticks have been identified, being the main genres *Anocentor, Dermacentor, Rhipicephalus, Hyalomma, Boophilus, Ixodes* and *Haemophysalis* (Scoles & Ueti, 2015). In Europe, *B.caballi* is transmitted mainly by ticks of the genre *Dermacentor* and *T.equi* by ticks of the genre *Rhipicephalus* and *Hyalomma*. The species present in Italy and Spain are *Dermacentor marginatus, Rhipicephalus bursa, Riphicephalus turanicus* and *Hyalomma marginatum*. In addition, *Hyalomma lusitanicum* is present in Spain (Habela *et al.*, 1989; Iori *et al.*, 2010).

Babesia caballi is found in different organs of ticks that may act as transstadial vectors. In addition, 8 of these species are also capable of transmitting *B.caballi* transovarianly. *Theileria equi* develops in the salivary glands, saliva, intestine and hemolymph of ticks (Mehlhorn and Schein, 1998), causing structural alterations of the salivary glands in some cases (Kumar *et al.*, 2007). Unlike *B.caballi*, there has not been evidence of trans-ovarian transmission in the case of *T.equi*, being only known the transstadial passage. When infected ticks

(Pfeifer Barbosa *et al*, 1995). Sono state identificate da 12 a 14 specie di zecche vettori, i generi principali *Anocentor, Dermacentor, Rhipicephalus, Hyalomma, Boophilus, Ixodes* e *Haemophysalis* (Scoles e Ueti*,* 2015). In Europa, *B.caballi* si trasmette principalmente tramite zecche del genere *Dermacentor,* e *T.equi* tramite zecche del genere *Rhipicephalus* e *Hyalomma*; le specie presenti in Italia e Spagna sono *Dermacentor marginatus, Rhipicephalus bursa, Riphicephalus turanicus* e *Hyalomma marginatum;* In Spagna inoltre, vi è presente *Hyalomma lusitanicum (*Habela *et al.* 1989; Iori *et al.* 2010).

*Babesia caballi s*i trova in differenti organi della zecca ed esse possono agire come vettori transtadiali; inoltre, 8 di queste specie sono anche in grado di trasmettere *B.caballi* di forma transovarica. *Theileria equi* si sviluppa nelle ghiandole salivari, saliva, intestino ed emolinfa della zecca (Mehlhorn e Schein, 1998), riscontrandosi alterazioni strutturali delle ghiandole salivari della zecca infettata con *T.equi* causate dal parassita (Kumar *et al.,* 2007). A differenza di *B.caballi* in *T.equi* non c'è evidenza di passaggio transovarico

feed on the equine host, sporozoites are inoculated. The sporozoites of *Babesia caballi* invade the erythrocytes and become trophozoites that grow and divide each into two merozoites of oval or piriform form. Those are able to infect other red blood cells repeating the division process. As for *Theileria equi*, inoculated sporozoites invade the lymphocytes, and the intraleucocitarian forms develop into schizonts. These will release merozoites that invade erythrocytes, transforming into trophozoites that grow and divide into pear-shaped infecting merozoites (that arrange in a Maltese cross form) inside the red blood cell.

Differences between *Babesia* and *Theileria* are based on the biological cycle (with a pre-erotrocytic phase in *T.equi*), the vectors and route of transmission (transstadial and transovarian or only transstadial), the morphological differences and the different susceptibility to drugs used for treatment. These differences made Mehlhorn and Schein in 1998 reclassify *Babesia equi* as *Theileria equi*.

A low specificity between parasite and host species caused in turn by the low

ma solo transtadiale.

Quando le zecche infette si alimentano dall'equino ospite, gli inoculano sporozoiti; gli sporozoiti di *Babesia caballi* invadono gli eritrociti e si trasformano in trofozoiti che crescono dividendosi ognuno in due merozoiti di forma ovale o piriforme che sono in grado di infettare altri globuli rossi ripetendo il processo di divisione. Nel caso di *Theileria equi* gli sporozoiti inoculati invadono i linfociti e questa forma intraleucocitaria si sviluppa formando schizonti, che rilasceranno merozoiti che invadono gli eritrociti trasformandosi in trofozoiti che crescono e si dividono in merozoiti infettanti con forma di pera che si dispongono in tetrade a modo di croce di Malta nel globulo rosso.

Le differenze tra *Babesia* e *Theileria* si basano sul ciclo biologico (presenza di una fase pre-eritrocitica in *T .equi*), i vettori che le trasmettono e le modalità (transtadiale e transovarica o solo transtadiale), così come la differenza morfologica e la differente suscettibilità ai farmaci usati per il trattamento, che indussero Mehlhorn e Schein nel 1998 a riclassificare *Babesia equi* come *Theileria*

specificity of the vector (Navarrete *et al.*, 1999) can be determined by the presence of piroplasms in other animal species. Thus, there have been isolated other parasites in horses such as *Encephalitozoon* (Ribeiro *et al*., 2006), *Babesia canis canis* (Criado-Fornelio *et al.,* 2003; Fritz*,* 2010; Zanet *et al.,* 2017), *Babesia canis rossi* (Fritz, 2010), *Babesia bovis* in asymptomatic animals (Criado *et al.*, 2006), *Theileria annae*, *Theileria sergenti* and *Theileria buffeli* (Moretti *et al.*, 2010). On the contrary, equine piroplasms have also been found in other species such as dogs and dromedaries for *Theileria equi* and *Babesia caballi* (Criado-Fornelio *et al*., 2003; Beck *et al.*, 2009; Fritz, 2010; Qablan *et al.*, 2012).

There have been discovered more than 30 species of piroplasms belonging to the genres *Theileria* and *Babesia* (Criado Fornelio *et al.*, 2004). According to Criado-Fornelio *et al.* (2003), the evolution of Piroplasmids from placental mammals could have happened in Africa from 55 to 20 million years ago. Due to its primitive characteristics, *T. equi* could be the ancestor of current Theilerids. In his study, Criado-Fornelio describes african *T. equi* as an ancestor of the Spanish varieties, and *equi*.

È stata osservata una discreta specificità tra il parassita e la specie ospite causata a sua volta per la bassa specificità del vettore (Navarrete *et al.,* 1999) questo può determinare la presenza in una specie animale di piroplasmi diversa di quella propria della specie; sono state isolate in cavalli altri parassiti come *Encephalitozoon* (Ribeiro *et al*., 2006), *Babesia canis canis* (Criado-Fornelio *et al.,* 2003; Fritz, 2010; Zanet *et al.,* 2017), *Babesia canis rossi* (Fritz, 2010), *Babesia bovis* in animali sintomatici (Criado *et al.,* 2006), *Theileria annae, Theileria sergenti* e *Theileria buffeli* (Moretti *et al.,* 2010). Al contrario si sono anche riscontrati piroplasmi equini in altre specie come *Theileria equi* o *Babesia caballi* in cani e dromedari (Criado-Fornelio *et al.,* 2003; Beck *et al.,* 2009; Fritz*,* 2010; Qablan *et al.,* 2012)

Sono state scoperte più di 30 specie di piroplasmi appartenenti ai generi *Theileria* e *Babesia* (Criado Fornelio *et al.,* 2004). Secondo Criado-Fornelio *et al.* (2003), l'evoluzione dei piroplasmidi dei mammiferi placentati potrebbe essere iniziata in Africa da 55 a 20 milioni di anni fa; *T.equi* potrebbe essere l'antenato degli

indicates that *B. caballi* or its ancestors would be the origin of a lineage of ungulibabesids of the horse, having passed the group to the primitive bovids and from there to the caprids.

To study the different genotypes, DNA amplification techniques such as PCR, reverse line blot (RLB) and sequencing from different molds have been used. First genotypic characterizations were carried out in Spain by Nagore *et al.* (2004), using the technique of reverse line blot and targeting the 18S, that helped to identify forms called "like" in addition to the classic ones in both genders.

18S rRNA subunit has been used in numerous studies because it has both conserved and variable regions, allowing for unequivocal alignment and subsequent phylogenetic discrimination. Other subunits have been poorly used, such as 28S, as it is too long and less informative for phylogenetic use. Other subunits like 5S and 8S have been discarded because they are too short. In addition to the ribosomal sequences, target sequences of the merozoite antigen (EMA-1) from *T. equi* (Bhoora *et al.*, 2010; Munkhjargal *et al.*, 2013; Ketter-Ratzon *et al.* 2017) and of the

attuali Theileridae viste le loro caratteristiche primitive; nei suoi studi descrive la *T.equi* africana come antenata delle varietà spagnole, e indica che *B. caballi* o i suoi antenati sarebbero l'origine di un lignaggio di ungulibabesidi del cavallo, essendo passato il guppo agli antenati dei bovidi primitivi e da lì ai capridi.

Per studiare i diversi genotipi sono state utilizzate tecniche di DNA, come PCR, reverse line blot (RLB) e sequenziamenti a partire da diversi stampi. Le prime caratterizzazioni genotipiche, furono realizzate in Spagna da Nagore *et al.* (2004), usando la tecnica di RLB e utilizzando come target il 18S col quale si identificò una forma chiamata “like” nonché le forme classiche in entrambi i generi.

La subunità del 18S rRNA è stata usata in numerosi studi perché presenta regioni conservate e variabili e questo permette allineamenti inequivoci e una posteriore discriminazione filogenetica; altre subunità sono state poco utilizzate, come la 28S per esempio, perche è troppo lunga e poco informativa per l'uso filogenetico, mentre le subunità 5S e 8S

associated-protein gene rhoptry 1 (RAP-1) from *B.caballi* (Bhoora *et al.*, 2010; Rapoport *et al.*, 2014) that have shown great heterogeneity (although the variability present in *B.caballi* seems inferior, probably due to the smaller number of positive samples found), and low levels of parasitemia hinder the genotypic classification. Other less used are the β-tubulin gene used for discrimination between species (Cacciò *et al.*, 2000) and cytochrome b genes (Criado *et al.*, 2006).

Studies using 18S rRNA, EMA and RAP, have identified differentiated clades in each genre. 18S has identified 3 to 5 groups for *T. equi* and two clades (one with two subgroups) for *B. caballi*; classic and "like" forms described by Nagore *et al.* (2014) appear in different clades. There were evidenced three clades using the EMA antigen and two using the RAP (one with two subclades). Sequences of the same samples analyzed using the 18S and a species specific target do not show an exact correspondence between clades identified with the different methods. These studies have been developed in different countries, and do not show geographical influence in the presence of the different genotypes

sono state scartate perché troppo corte.

Oltre alle sequenze ribosomiali sono state usate sequenze target dell'antigene del merozoito (EMA-1) di *T.equi* (Bhoora *et al.,* 2010; Munkhjargal *et al.,* 2013; Ketter-Ratzon *et al.,* 2017), e del gene della proteina associata al rhoptry 1 (RAP-1) di *B.caballi* (Bhoora *et al.,* 2010; Rapoport *et al.,* 2014) che hanno mostrato una grande eterogenicità sebbene la variabilità presente in *B.caballi* sembrerebbe inferiore dovuto probabilmente al minor numero di campioni positivi riscontrati, e al basso livello di parassitemia che rendono difficile la classificazione genotipica. Altre meno utilizzate sono state il gene della β-tubulina usato per la discriminazione tra specie (Cacciò *et al.,* 2000) e il gene del citocromo b (Criado *et al.,* 2006).

Negli studi in cui si è usato il 18S rRNA, EMA e RAP sono stati identificati cladi differenziati presenti in ogni genere, con il 18S si sono identificati da 3 a 5 gruppi per *T.equi* e due cladi (uno con due sottogruppi) per *B.caballi* la forma classica e la "like" descritta da Nagore *et al.* (2014) appaiono in cladi differenti. Usando l'antigene EMA sono stati evidenziati tre

since the presence of these subgroups is maintained. There have been no studies on pathogenicity or any identification of more virulent genotypes.

Geographical distribution is linked to the vector, although clinical cases of piroplasmosis have been described worldwide. The disease is endemic in tropical, subtropical and temperate areas (Brüning, 1996). Iceland, England and Ireland, Australia, Japan, Russia, Canada and the United States (except Florida and Texas) are not considered endemic areas. In Australia, the disease was introduced in the mid-1970s by importation of positive Spanish horses but was not established in the native population (Mahoney *et al.*, 1977). In southern United States it was introduced through Cuban horses imported in 1959, and *Dermacentor nitens* vector transmitted it to native horses. Some theoretically free areas have shown seropositive animals and vectors, suspected to have been transported by wild animals or migratory birds. (Butler *et al.*, 2012).

In Mediterranean countries, infections caused by *T. equi* are more frequent than those caused by *B. caballi*

cladi e con il RAP due (uno presenta due subcladi). Le sequenze ottenute dallo stesso campione analizzato usando il 18S ed un target specifico di specie non mostrano una esatta corrispondenza tra i cladi identificati con i diversi metodi. Questi studi sono stati svolti in differenti paesi e non si evidenzia un'influenza geografica nella presenza dei differenti genotipi visto che la presenza di questo sottogruppo rimane. Non sono stati realizzati studi sulla patogenicità né identificati genotipi più virulenti.

La distribuzione è legata alla presenza del vettore e anche se sono stati descritti casi clinici di piroplasmosi a livello mondiale, la malattia è endemica nell'area tropicale, subtropicale e in zone temperate (Brüning, 1996). Non si considerano zone endemiche l'Islanda, Inghilterra e Irlanda, Australia, Giappone, Russia, Canada e Stati Uniti (eccetto Florida e Texas). In Australia si introdusse a metà degli anni 70 con l'importazione di cavalli spagnoli positivi però la malattia non si stabilì nella popolazione autoctona (Mahoney *et al.*, 1977), e nel sud degli Stati Uniti si introdusse con cavalli cubani importati nel 1959 e il vettore *Dermacentor nitens* la trasmise ai cavalli nativi. In alcune zone

(Bashiruddin *et al.*, 1999). However, *B. caballi* is more prevalent in Africa.

The table shows the prevalence in the Mediterranean countries in the past and present century:

In endemic areas, colts receive maternal antibodies with colostrum (de Waal and Van Heerden 1994), which can be maintained up to five or six months of age and confer protection during this period (de Waal and Van Heerden, 2004). In a study conducted in Spain by Coleto in 1999, 90% of foals born from positive mares remained IFAT-positive for 90-100 days. Colts born in endemic areas usually present subclinical infections. Maternal immunity is gradually replaced by active and stable immunity due to the continued presence of the parasite (Phipps *et al.*, 1996).

Animals that have never been in contact with the parasite and become infected develop antibodies approximately ten days after post-infection. Antibodies against *B. caballi* usually last about four years. In the case of *T. equi*, developed antibodies remain for life (De Waal, 1992). Immunodominant surface antigens of merozoites are an important target for the

teoricamente indenni, sono stati trovati animali sieropositivi e si sospetta che il vettore sia stato portato tramite animali selvaggi o uccelli migratori (Butler *et al.,* 2012).

Nel bacino mediterraneo le infezioni causate da *T. equi* sono più frequenti di quelle dovute a *B. caballi* (Bashiruddin *et al.,* 1999; Criado-Fornelio *et al.,* 2003) tuttavia in Africa *B. caballi* è più prevalente.

Nella tabella è indicata la prevalenza di alcuni studi effettuati nel bacino mediterraneo nel secolo passato e in quello attuale.

Nelle zone endemiche, i puledri ricevono anticorpi materni con il colostro (De Waal e Van Heerden, 1994) che si possono mantenere fino ai 5 o 6 mesi di età e che gli conferisce protezione durante questo periodo (de Waal e Van Heerden*.,* 2004). In uno studio realizzato in Spagna da Coleto nel 1999, il 90% dei puledri nati da madri positive rimangono positivi in IFI durante 90-100 giorni. I puledri nati in zone endemiche spesso presentano infezioni subcliniche, poiché l'immunità materna si sostituisce gradualmente con un'immunita attiva e stabile dovuta alla continua

protective immune response, and many ELISA tests and vaccine trials have used epitopes of these proteins. They are the RAP-1 proteins in *Babesia* and the EMA in *T.equi*. EMA-1 and EMA-2 are not expressed during stages of asexual erythrocyte development. They are only expressed during the first phase of sexual development (Kumar *et al.,* 2004).

The incubation period of *Theileria equi* in horses is 12 to 19 days (Mehlhorn and Schein, 1998), when merozoites are observed in blood smears. However, schizonts inside lymphocytes are found two days after infection. Parasitemia can be high, becoming infected up to 80% of the erythrocytes in splenectomized animals (de Waal and van Heerden, 2004). In *Babesia caballi* infections, the incubation period lasts from 10 to 30 days and parasitemia is much lower than that observed in cases of theileriosis, around 1%. In immunocompromised animals, incubation times and prepatencies are shortened, and the severity of the disease increased (Wise *et al.*, 2014).

Equine piroplasmosis is a hemolytic febrile syndrome. Fever coincides with the sudden release of hemoglobin and the final

presenza del parassita (Phipps *et al.,* 1996).

Negli animali che non sono stati in contatto col parassita e si infettano, la comparsa di anticorpi si produce approssimamente dieci giorni dopo l'infezione, e nel caso di *B. caballi* gli anticorpi persistono un quattro anni, e in *T. equi* tutta la vita dell'animale. (de Waal, 1992).

Gli antigeni immunodominanti della superficie dei merozoiti sono un target importante per la risposta immune protettrice e per questo in molti test ELISA e prove di vaccini hanno usato epitopi di questa proteina. Queste sono la proteina RAP-1 in *Babesia* e EMA in *T.equi.* EMA-1 e EMA-2 non si esprimono durante lo stadio di sviluppo eritrocitario asessuale espremendosi solo durante la prima fase di sviluppo sessuale (Kumar *et al.*, 2004).

Il periodo di incubazione di *Theileria equi* negli equini va dai 12 ai 19 giorni (Mehlhorn e Schein, 1998) fino a che i merozoiti si osservano nello striscio sanguigno, tuttavia gli schizonti dei linfociti si riscontrano due giorni dopo l'infezione. La parassitemia può essere elevata arrivando ad osservare fino a un 80% di eritrociti infettati in animali

products of erythrocyte lysis. Hemolysis is the result of mechanical damage of erythrocytes due to intra-erythrocyte binary fission of trophozoites, toxic damage caused by haemolytic factors produced by the parasite, immunomediation of direct autoantibodies against components of the membranes of infected and non-infected red blood cells, and the toxicity of hemolytic factors released by the piroplasm (Mahmoud *et al.*, 2016). The pathogenic action of these parasites is also based on the release of pharmacologically active substances (esterases) that activate kallikrein, a biogenic amine that causes circulatory alterations, vasodilatation, increased vascular permeability, circulatory stasis, shock and death. In the case of *T.equi*, the parasite focuses its action on the phagocytic mononuclear system (lymphoproliferative phase), and causes severe anemia with high parasitemia (Ambawat *et al.*, 1999). The role of EMA antigens (*T.equi*) and host sialic acids in the binding site to the erythrocyte has been determined by observing, in the case *B.caballi*, that by removing or blocking the sialic acids from the erythrocyte membrane a minor number of parasites manage to invade the cell by finding more merozoites

splenetomizzati (de Waal e van Heerden, 2004). In *Babesia caballi* il periodo di incubazione va dai 10 a 30 giorni, la parassitemia è molto minore di quella osservata in casi di theileriosi e si riscontrano valori intorno all'1 %. In animali immunodepressi i tempi di incubazione e prepatenza si accorciano, e la gravità della malattia aumenta (Wise *et al.,* 2014).

La piroplasmosi equina è una sindrome febbrile emolitica; la febbre coincide con la liberazione repentina di emoglobina e del prodotto finale della lisi eritrocitaria. L'emolisi è il risultato del danno meccanico degli eritrociti dovuto alla fissione binaria intraeritrocitaria dei trofozoiti, del danno tossico causato da fattori emolitici prodotti dal parassita e immunomediato da autoanticorpi diretti contro i componenti della membrana dei globuli rossi infettati e non infettati e della tossicità del fattore emolitico che libera il piroplasma (Mahmoud *et al.,* 2016). L'azione patogena di questi parassiti si basa anche nella liberazione di sostanze farmacologicamente attive (esterase) che attivano la calicreina, una amina biogena che produce alterazione circolatoria, vasodilatazione, incremento della permeabilità vascolare, stasi circolatoria,

outside the erythrocytes while reducing the growth of babesias. In the same experiments with *T.equi*, intracellular development of the parasites is altered (Okamura *et al.*, 2005). Hanafusa *et al.* in 1998, studied the role of some cytokines such as γIFN, αTNF and IL-2 (related to pyrexia), as well as nitric oxide, an indicator of endothelial tissue damage and edema. These cytokines control and reduce parasitaemia, but if the equilibrium is broken they can cause renal damage and aggravate endotoxic shock during septicemia. TNF and NO can help to control and reduce parasitemia but if the equilibrium breaks or an excessive increase of levels happens they cause severe damage.

The function of the spleen in the control of piroplasmosis is based on its phagocytic activity, the antibody response against circulating antigens and its ability to counteract genetic variation.

Equine piroplasmosis presents 4 clinical phases:

-Hyperacute phase: is a rare form in which animals appear dying or dead.

-Acute phase: is the most common

shock e morte. Nel caso di *T.equi* il parassita centra la sua azione sul sistema mononucleare fagocitario (fase linfoproliferativa) e dopo causa un'anemia severa con alta parassitemia (Ambawat *et al.,* 1999). È stato determinato il ruolo degli antigeni EMA (*T. equi)* e dell'acido sialico dell'ospite nell'unione del parassita con l'eritrocito, osservando che eliminando o bloccando l'acido sialico della membrana eritrocitaria nel caso di *B.caballi,* un minor numero di parassiti riescono ad invadere la cellula riscontrando più merozoiti fuori dall'eritrocito mentre si riduce l'accrescimento della babesia. Nello stesso esperimento con *T.equi* lo sviluppo intracellulare dei parassiti si osserva alterato (Okamura *et al.*, 2005). Hanafusa *et al.* nel 1998 studiarono l'azione di alcune citochine come le γIFN, αTNF e IL-2 (relazionate con la piressia), così come l'ossido nitrico, indicatore di danno tessutale dell'endotelio e edema. Queste citochine agiscono per controllare e ridurre la parassitemia, però se si rompe l'equilibrio possono causare danni renali e aggravare lo shock endotossico durante la setticemia. TNF e NO possono aiutare a controllare e ridurre la parassitemia però se si rompe l'equilibrio o aumentano

form, animals present high fever (coinciding with high parasitemia), weakness, anorexia and discomfort, increased heart rate, congestive, anemic or icteric mucous membranes, dark-colored urine and small, dry feces.

- Subacute phase: similar to the acute phase, animals also present weight loss and intermittent fever. Colour of the mucous membranes ranges from pale pink to pink or light yellow to bright yellow, petechiae and ecchymosis, as well as edema in the distal parts of the extremities, may also occur. Intestinal motility may be affected and signs of colic may be observed.

- Chronic phase: in these cases, animals present nonspecific clinical signs such as anorexia, poor performance and lower body condition. This form is very frequent in donkeys, and it is associated to low parasitemia.

Other clinical signs observed are depression, intermittent fever, anorexia, rhinorrhea, sialorrhea, lameness and encephalitis (in infections caused by *Babesia*), bronchopneumonic symptoms, edema of the head and eyelids,

eccesivamente causano gravi danni.

La funzione della milza nel controllo della piroplasmosi si basa sulla sua attivita fagocitica, e nella risposta anticorpale contro gli antigeni in circolazione e nella sua capacità di contrastare la variazione genetica.

La piroplasmosi equina presenta le seguenti forme cliniche:

-Iperacuta: è una forma poco frequente dove l'animale appare moribondo o morto.

-Acuta: è la forma più comune, gli animali presentano febbre alta (coincidendo con l'alta parassitemia), debolezza, scomparsa di appetito e malessere, aumento della frequenza cardiaca, mucose congestive, anemiche o itteriche e si osservano le urine di colore scuro e feci piccole e secche.

-Subacuta: è simile all'acuta ma accompagnata da perdita di peso e febbre intermittente; il colore delle mucose va dal rosa pallido al rosa o dal giallo chiaro al giallo brillante, si possono manifestare petecchie e echimosi così come edemi nella parte distale delle estremità. La motilità intestinale può essere alterata e si osservano nell' animale segni di colica.

lacrimation, conjunctivitis, incoordination and enlarged lymph nodes.

Non-symptomatic carriers may relapse under stress conditions or immunosuppression (Wise *et al.*, 2014). Clinical forms caused by *T.equi* are more severe than those caused by *B.caballi*, and the mortality rate is higher. *T.equi* can also cause "in utero" infections causing abortions or foals with neonatal piroplasmosis. Some foals may be born as healthy carriers, where the immune system does not recognize the parasite as strange and the antibodies acquired with the colostrum would control the levels of parasitemia during the first months of life. (Georges *et al.*, 2011, Sant *et al.,* 2016).

Lesions observed are linked to hemolysis: the blood is diluted or watery, mucous membranes appear pale or icteric, and dark red blood is frequently seen in urine. There may be slight hepatomegaly, and sometimes degeneration and coagulative necrosis may be observed. The spleen shows splenomegaly with friable tissue and dark coloration. The kidneys are the most affected organs, presenting a darker or clearer coloration than normal with severe congestion in the marrow area

-Cronica: in questo caso, l'animale presenta segni clinici non specifici come inappetenza, basso rendimento e minor condizione corporale. Questa forma clínica è molto frequente nell'asino e va associata ad una bassa parassitemia.

Altri segni clinici osservati sono depressione, febbre intermittente, anoressia, rinorrea, sialorrea, zoppia e encefaliti in infezione causata da *Babesia;* sintomi broncopneumonici, edema sulla testa e palpebre, lacrimazione, congiuntivite, incoordinazione e linfonodi aumentati.

Gli individui apparentemente sani che sono portatori possono recidivare in certe condizioni di stress o immunodepressione (Wise *et al.,* 2014). Le forme cliniche causate da *T.equi* sono più gravi di quelle causate da *B.caballi* e il tasso di mortalita è piu elevato, *T.equi* inoltre può causare infezioni “in utero” provocando aborti o puledri con piroplasmosi neonatale, alcuni possono nascere come portatori sani, e in questi ultimi il sistema immune non riconosce come estraneo il parassita mentre gli anticorpi acquisiti con il colostro controllano il livello di parassitemia durante il primo mese di vita

where petechiae can be observed. In the heart, frequent hemorrhages are observed in the epicardium, myocardium and endocardium. There is also vacuolar degeneration and congestion with separation of the myocardial muscle bundles. The lungs may present acute congestion, edema and thrombosis of the pulmonary vessels. Secondary infections can lead to minor lung lesions such as emphysema or pneumonia. Other lesions described are subcutaneous edema, visceral congestion, and presence of fluid in the pericardium and peritoneum (Hailat *et al.*, 1997, Hanafusa *et al.*, 1998; Kumar *et al.*, 2009).

Most frequent alterations of red cells are: low erythrocyte count, hemoglobin and hematocrit, and relative variations in RDW, MCV, MCH and MCHC that occur in the course of microcytic and hypochromic anemia. The activity of the hematopoietic organs can also be altered by releasing poikilocytes and macroeritrocytes (Mahmoud *et al.*, 2016; Sumbria *et al.*, 2017). As for the white cells, the authors' observations have been variable finding cases of leukopenia and leukocytosis, neutropenia and

dell' animale. (Georges *et al.,* 2011; Sant *et al.,* 2016).

Le lesioni osservate vanno legate all'emolisi: il sangue si presenta diliuito o acquoso e la mucose pallide o itteriche, inoltre si riscontra frequentemente sangue di colore rosso scuro nella vescica. Può presentarsi una leggera epatomegalia, e in alcuni casi si puo osservare degenerazione e necrosi coagulativa. La milza presenta esplenomegalia con una colorazione molto scura e friabile. I reni sono gli organi più colpiti presentando una colorazione molto più scura o chiara del normale con congestione grave, e nella zona midollare si possono osservare petecchie. Nel cuore si osservano frequenti emorragie nell'epicardio, miocardio e endocardio, degenerazione vacuolare e congestione con una certa separazione delle fasce muscolari miocardiche. I polmoni possono presentare congestione acuta, edema e trombosi dei vasi polmonari; le infezioni secondarie possono produrre lesioni polmonari poco specifiche come enfisema o polmonite. Altre lesioni descritte sono: edema sottocutaneo, congestione viscerale e presenza di liquido nella pleura, pericardio e peritoneo (Hailat *et al.,* 1997;

neutrophilia, lymphopenia or lymphocytosis (Al-Saad *et al.*, 2009: Laus *et al.*, 2015) and monocytosis (Diana *et al.,* 2007). Platelet count is low, resulting from local and systemic disseminated intravascular coagulation, immune-mediated destruction and blocking of platelets in the spleen (Beard *et al.*, 2013). Other alterations in coagulation parameters such as plasma fibrinogen, D-dimer, coagulation time, prothrombin time, activated partial thromboplastin have been described by different authors (Mantran et al., 2004, A-Saad *et al.,* 2009).

Other frequent findings are increased bilirubin caused by hemolysis (Al-Saad *et al.*, 2009), elevated hepatic transaminases due to central-lobular degeneration and hepatocyte necrosis (Camacho *et al.*, 2005; Zobba *et al.*, 2008), and hypoproteinemia with hypoalbuminemia and alterations in α2- and γ- globulins (Rubino *et al.* 2006; Barrera *et al.*, 2010). It has also been observed the presence of markers for cardiac damage due to a possible myocardial alteration as well as increased CK due to muscle damage (Diana *et al.*, 2007). High values of oxidative stress and lipid peroxidation markers such

Hanafusa *et al.,* 1998; Kumar *et al.,* 2009).

Le alterazioni della serie rossa più frequenti sono: conto eritrocitario, emoglobina e ematocrito bassi, variazioni relative in RDW, MCV, MCH e MCHC che si producono nel corso di anemia microcitica e ipocromica. Inoltre puo alterarsi l'attività degli organi ematopoietici liberandosi poichilociti e macroeritrociti (Mahmoud *et al.,* 2016; Sumbria *et al.,* 2017). In quanto alla serie bianca le osservazioni degli autori sono variabili, riscontrando casi di leucopenia e leucocitosi, neutropenia e neutrofilia, linfopenia o linfocitosi (Al-Saad *et al.,* 2009; Laus *et al.,* 2015) e monocitosi (Diana *et al.,* 2007). Il conto delle piastrine è basso, conseguenza della coagulazione intravascolare disseminata locale e sistemica, la distruzione immunomediata e sequestro delle piastrine dalla milza (Beard *et al.,* 2013). Altre alterazioni nei parametri della coagulazione come il fibrinogeno plasmatico, D-dimero, tempo di coagulazione, tempo di protrombina, tromboplastina parziale attivata sono state descritte da differenti autori (Mantran *et al.,* 2004; A-Saad *et al.,* 2009).

Altri risultati frequenti sono l'aumento della bilirubina causato

as malondialdehyde, as well as high levels of serum glutathione and low levels of vitamin E have also been reported (Ambawat *et al*., 1999; Deger *et al.,* 2009).

The risk factors associated with equine piroplasmosis recently described by different authors (García-Bocanegra *et al.*, 2012; Bartolomé del Pino *et al.*, 2016, Zanet *et al.*, 2017, Sumbria *et al.*, 2017) indicate the influence of individual characteristics such as species, age, gender, race and aptitude. There is a higher prevalence of the disease in mules and donkeys, in females, in adult and geriatric animals due to the persistence of antibodies, and in animals of native and crossed breeds, maybe due to differences in the susceptibility of those to the disease. Other risk factors that have been are related to management practices are: type of accommodation and access to the grass that determine a higher contact with vectors, and administration of anti-parasitic treatments and regular vaccinations defined as protective factors and being an indicator of good practices. Different factors related to the ecology of the vector such as geographical area, height, climate, soil type, and land cover,

dall'emolisi (Al-Saad *et al.,* 2009); transaminasi epatiche elevate per la degenerazione centrolobulare e necrosi dell'epatocito (Camacho *et al.,* 2005; Zobba *et al.,* 2008) e la ipoproteinemia con ipoalbuminemia, alterazioni nelle globuline α2 e γ globuline (Rubino *et al.,* 2006; Barrera *et al.,* 2010).

Sono state osservate inoltre la presenza di marcatori di danno cardiaco dovuto ad una possibile alterazione miocardica così come la CK aumentata per il danno muscolare (Diana *et al.,* 2007)

Inoltre sono stati riportati alti valori di marcatori di stress ossidativo e perossidazione lipidica come il malondialdeide così come alti livelli di glutatione sierico e bassi livelli di vitamina E (Ambawat et al., 1999; Deger *et al.,* 2009).

I fattori di rischio associati alla piroplasmosi equina recentemente descritti da differenti autori (García-Bocanegra *et al.,* 2012; Bartolomé del Pino *et al.,* 2016; Zanet *et al.,* 2017; Sumbria *et al.,* 2017) segnalano l'influenza di caratteristiche individuali degli equini come specie, età, genere, razza e attitudine osservando una maggiore prevalenza in

have been observed. In general, these factors refer to conditions favoring the presence of vectors and their contact with susceptible animals.

There are different types of diagnosis:

- Clinical-epidemiological, based on symptomatology, presence of ticks, foci of the disease, etc. This type of diagnosis is not very specific and should always be corroborated with a laboratory technique.

-The differential diagnosis contemplates different diseases that occur with hemolytic anemia and / or jaundice such as ehrlichiosis, equine infectious anemia, African horse sickness, trypanosomiasis, leptospirosis, intoxications, arteritis, hemorrhagic purpura, etc ...

-Direct Parasitological diagnosis: consists of directly evidencing the piroplasms in blood smears or by PCR.

-Indirect Parasitological diagnosis: based on the determination of antibodies. The most representative are complement fixation, immunofluorescence and ELISA.

Regarding direct parasitological

muli e asini, nelle femmine, in animali adulti e anziani per la persistenza di anticorpi, e in animali di razza autoctona e incrociata dovuto a differenze nella suscettibilità delle stesse alla malattia. Altri fattori di rischio evidenziati in questi studii sono relazionati col maneggio degli animali; il tipo di alloggio e l' acceso al pascolo che determinano un maggior contatto con i vettori. L'amministrazione dei trattamenti repellenti, gli antiparassitari interni e le vaccinazioni regolari sono stati definiti come fattori protettori indicatori di buone pratiche. Sono stati osservati diversi fattori relazionati con l'ecologia del vettore come: area geografica, altezza, clima, tipo di suolo o copertura terrestre.

In generale, questi fattori fanno riferimento a condizioni che favoriscono la presenza dei vettori e un maggior contatto degli animali con essi.

Esistono diverse modalità di metodi diagnostici:

-Clinico-epidemiologico, basato sulla sintomatologia, presenza di zecche, focolai di malattia nelle vicinanze, ecc. Questo tipo di diagnostico è poco specifico e sempre si deve corroborare con una

techniques:

Blood smears are usually made by extracting blood from superficial capillaries (snout or ears) during the acute phase of the disease (feverish peak). They can also be made from imprints of organs during necropsies (brain, kidney, liver, lungs). These smears are stained with Giemsa or Diff-Quick and are observed under immersion by optical microscopy. This technique presents some limitations, such as difficulty identifying piroplasms according to the genre, identification in carrier animals or in cases of low parasitemia, the need for experienced operators and the lack of practicality in large scale studies.

-PCR is increasingly being used for diagnosis because of its high sensitivity and specificity. Commercial kits already exist. There are many experimental studies using different targets: constitutive genes or antigens from the different stages such as merozoites.

Regarding indirect techniques:

- Complement fixation is a very sensitive technique, and it is used for international movement and trade in some

tecnica di laboratorio.

-La diagnosi differenziale contempla diverse malattie che presentano segni clinici comuni come l'anemia e/o ittero di origine emolitica, ad esempio l'ehrlichiosi, anemia infettiva equina, peste equina, tripanosomiasi, leptospirosi, intossicazione, arterite, porpora emorragica ecc...

-Parassitologica diretta: consiste nell'evidenziare direttamente i piroplasmi nello striscio sanguigno o PCR.

-Parassitologica indiretta: si basa nella determinazione degli anticorpi, i più rappresentativi sono la fissazione del complemento, l'immunofluorescenza e l'ELISA.

In quanto alla tecnica parassitologica diretta:

-Lo striscio sanguigno di solito si fa estraendo sangue dai capillari superficiali (muso e orecchie) nella fase acuta della malattia (picco febbrile), si può anche preparare a partire da impronte di organi durante le necroscopie (cervello, reni, fegato e polmoni). Questo striscio si ottiene con Giemsa o Diff-Quick e si osserva con il microscopio ottico a immersione. Alcuni limiti di questa tecnica

countries, but the test does not identify all infected animals, especially if they have been treated because anti-complementary reactions occur.

- Immunofluorescence is one of the tests prescribed by the OIE; is a test with greater sensitivity than the previous one in chronic cases, but presents cross-reactions between the two protozoa (Friedhoff *et al.*, 1986; Brüning, 1996). Sometimes it is not easy to differentiate between weak positives and negatives, and a great interpretive experience is required.

-The competitive ELISA is an already commercialized test recommended by the OIE. A recombinant protein and a monoclonal antibody defining an epitope on the surface of the merozoite are used. The ELISA test is easy to develop and avoids the problem of cross reactions. It also allows testing many samples at a time. The main problem is that while the *T. equi* test covers many varieties distributed across different geographic areas, that of *B. caballi* does not seem to be able to identify certain variants of African origin that are also found elsewhere (Bhoora *et al.,* 2010; Rapoport *et al.*, 2014). Other types of indirect type ELISAs using recombinant

sono la difficoltà di identificare il piroplasma secondo il genere, l'identificazione in animali portatori o in caso di bassa parassitemia, la presenza di operatori esperti e la poca praticità in studi in grande scala.

-La PCR si usa sempre di più per la diagnostica grazie alla sua elevata sensibilità e specificità; sono presenti sul mercato kit commerciali. Ci sono molti studi sperimentali che usano diversi target: geni costitutivi e altri antigeni della forma di replicazione come il merozoito.

Sulle tecniche indirette:

-La fissazione del complemento mostra una grande sensibilita e ancora si usa per movimentazione internazionale in alcuni paesi, ma questo test non identifica tutti gli animali infettati, sopratutto se sono stati sottoposti a trattamento poiché si producono reazioni anticomplementari.

-La immunofluorescenza è una delle prove prescritte dalla OIE; è un test con maggior sensibilita della tecnica precedente in casi cronici, ma presenta reazioni incrociate fra i due protozoi (Friedhoff *et al.,* 1986; Brüning*.,* 1996), ed a volte non è facile differenziare tra deboli positivi e negativi e bisogna avere una gran

proteins have been developed experimentally but are not commercially available.

Other diagnostic techniques found in the literature are the western blot, the agglutination test in latex and the in vitro blood culture (Holman *et al*., 1997).

The treatment may have different targets depending on each case. In endemic countries it focuses on saving the animal's life and relieving the symptoms. Healthy carriers are not treated. However, in non-endemic areas the treatment seeks not only to combat the symptomatology of the animal but to sterilize it, thus preventing it from being a carrier and spreading the infection (Wise *et al.*, 2014).

The most frequent treatment used is intramuscular imidocarb dipionate. In donkeys, it is recommended not to pass the dose of 2 mg / kg, since this species has been observed to be more susceptible to adverse effects (Singh *et al.*, 1980). It is a very effective treatment, especially for *Babesia* infections. *Theileria* infections are more refractory, presenting differences in susceptibility between different varieties of *T.equi* (Hines *et al.*, 2015). It also has

esperienza interpretativa.

-L'ELISA competitivo è una prova già commercializzata che raccomanda la OIE. Si usa una proteina ricombinante e un anticorpo monoclonale che definisce un epitope della superficie del merozoito. Il test ELISA è di facile svolgimento, e si evita il problema della reazione crociata, e permette di testare molte campioni alla volta; il problema attualmente è che mentre il test di *T. equi* copre diverse varietà distribuite in diverse aree geografiche, il test di *B. caballi* sembra non riesca ad identificare certe varianti di origine africana, che si trovano in altri posti (Bhoora *et al.,* 2010; Rapoport *et al.,* 2014). È stato sviluppato sperimentalmente un altro tipo di ELISA di tipo indiretto che usa proteine ricombinanti ma non è stato commercializzato.

Altri metodi diagnostici trovati in letteratura sono il western blot, test di agglutinazione in latex e coltivazione di sangue in vitro (Holman *et al*., 1997).

Il trattamento potrebbe avere obiettivi differenti secondo il caso, in paesi endemici si centra nel salvare la vita dell'animale e alleviare i sintomi, e non si sottopongono a trattamento i portatori

undesirable side effects due to its activity anticholinesterase that can cause cramps, and it requires monitoring. To avoid these effects, atropine is sometimes added directly to the treatment (Abutarbush *et al.*, 2012). In many cases of theileriosis imidocarb is combined with an antibiotic such as tetracycline, to which *T. equi* is sensitive in the pre-erythrocytic phase. Other associations are imidocarb and buparvaquone (Rothschild, 2013).

In acute clinical cases a supportive treatment is usually necessary.

Nowadays, many countries follow the OIE recommendations for international movement of animals, requesting a certificate indicating the absence of symptoms on the day of shipment, seronegativity to the tests prescribed by the OIE 30 days before departure and a treatment against ixodids 7 days before shipment.

Some useful practices to prevent new infections are management measures related to transmitting vectors. Therefore, it is advised the use of external parasite drugs (repellents, acaricides), limiting exits to the grass, inspecting animals and

sani, invece nelle zone non endemiche il trattamento cerca non solo di combattere la sintomatologia dell'animale ma di sterilizzarlo evitando che possa divenire portatore e propagare l' infezione (Wise *et al.*, 2014).

Il trattamento più frequente è il dipionato di imidocarb per via intramuscolare. Negli asini, si consiglia di non superare la dose di 2 mg/kg poiché è stato osservato che questa specie è più suscettibile agli effetti avversi (Singh *et al.* 1980). È un trattamento abbastanza efficace, sopratutto nelle infezioni da *Babesia*, quelle da *Theileria* sono più refrattarie essendo state osservate differenze nella suscettibilità tra diverse varietà di *T.equi* (Hines *et al*., 2015). Il farmaco ha effetti secondari indesiderati dovuti alla sua attività anticolinesterasica che può provocare coliche richiedendo un certo monitoraggio. Per evitarlo, in alcune occasioni si inietta atropina direttamente insieme al trattamento (Abutarbush *et al.,* 2012). In molti casi di theileriosi si combina l'imidocarb con un antibiotico come la tetraciclina, alla cui *T. equi* è sensibile nella fase pre-eritrocitica; altre associazioni sono imidocarb e buparvaquone (Rothschild, 2013). In quadri clinici acuti può essere

controlling the environment to eliminate possible ticks.

It is also essential to avoid iatrogenic transmissions, avoiding the sharing of needles between animals, and using disposable materials.

In the case of suspect animals, it is advised to start a quarantine to avoid contagion, and to proceed to the diagnosis and treatment in case of positivity.

Vaccination is not yet possible, since effective vaccines have not been commercially marketed. However, some vaccines had been experimentally developed in donkeys with promising results (Kumar *et al.*, 2002).

necessario un trattamento di supporto.

Attualmente in molti paesi si seguono le raccomandazioni dell'OIE per il movimento internazionale di animali sollecitando un certificato dove si indica l'assenza di sintomi il giorno dell'imbarco, sieronegatività ai test prescritti dalla OIE 30 giorni prima di imbarcare e un trattamento contro Ixodidi effettuato 7 giorni prima dell'imbarco. Alcune pratiche utili per prevenire nuove infezioni sono le misure di maneggio relazionate con il vettore, per questo si consiglia l'uso di antiparassitari esterni (repellente, acaricida), limitare l'uscita al pascolo, ispezionare gli animali e controllare l'ambiente per eliminare possibili zecche. Inoltre è fondamentale evitare la via iatrogenica, evitando di condividere aghi tra gli animali, e usando materiale usa e getta. Nel caso di animali sospetti, si effettua una quarantena per evitare contagio e procedere al diagnostico e al trattamento in caso di positività.

La profilassi vaccinale ancora non è possibile visto che non sono stati sviluppati vaccini efficaci anche se sperimentalmente erano stati sviluppati vaccini in asini con risultati incoraggianti (Kumar *et al.* 2002) sebbene non ne sono stati valutati altri.

CAPÍTULO 1: TÉCNICAS DE DIAGNÓSTICO

CHAPTER 1: DIAGNOSTIC TECHNIQUES CAPITOLO 1: TECNICHE DIAGNOSTICHE

RESUMEN CAPÍTULO 1

En este capítulo se describen y comparan las técnicas de diagnóstico que se usarán durante todos los experimentos incluidos en esta memoria: el frotis sanguíneo, las pruebas serológicas como el ELISA o immunofluorescencia y diferentes tipos de PCR (protocolos completos descritos en Anexo I).

Las pruebas que la OIE aconseja para diagnóstico y movimiento internacional de animales son serológicas y esto presenta un límite en la detección de animales enfermos en los primeros estadios de la enfermedad, cuando aún no se han desarrollado anticuerpos específicos y en la distinción entre animales portadores y seropositivos.

En este estudio, se seleccionaron 103 muestras de sangre de équidos sintomáticos y se efectuaron ocho tipos de PCR, cuatro distintas para cada tipo de piroplasma; PCR de punto final, anidada y PCR en tiempo real adaptada de un protocolo descrito en literatura y un kit comercial. También se prepararon frotis sanguíneos y se realizaron los test serológicos descritos por la OIE (ELISA e IFI). Las muestras discordantes se secuenciaron para observar la especificidad de los resultados.

Para ambos parásitos, las PCR más específicas fueron los protocolos adaptados y se usaron como referente para calcular la sensibilidad y especificidad relativas (rSe, rSp) y se calculó el porcentaje de acuerdo observado entre técnicas. También se utilizó el test de Chi cuadrado para evidenciar diferencias significativas entre los resultados de las PCRs y entre los métodos directos usados (PCR y frotis), así como entre los indirectos (IFI y ELISA).

Entre las técnicas para diagnosticar *B. caballi*, la técnica que mostró mayor rSe fue el frotis y la que presentó mayor rSp fue el ELISA. En cuanto a las técnicas de *T. equi* la de mayor rSe fue la IFI y la de mayor rSp el frotis. El porcentaje de acuerdo observado entre las PCR en tiempo real y las otras otras técnicas osciló del 78 al 96%. Se observaron diferencias estadísticamente significativas entre los dos métodos directos y entre los dos indirectos, sin embargo no se observaron entre las pruebas de PCR.

A la vista de los resultados se considera necesario añadir una técnica de diagnóstico molecular a las pruebas serológicas para diagnosticar adecuadamente la piroplasmosis equina y conseguir identificar portadores entre los seropositivos así, como animales recientemente

infectados.

Contribución de la autora: elaboración de todas las PCRs excepto los kits comerciales, extracción y purificación del DNA para secuenciación; el 30% de los análisis IFI; cálculos de los valores de rSe, rSp y acuerdo observado entre técnicas y redacción del escrito.

RIASSUNTO CAPITOLO 1

In questo capitolo si descrivono e si comparano le tecniche diagnostiche che sono state usate in tutti gli sperimenti della tesi: lo striscio sanguigno, le prove sierologiche come l'ELISA o l'immunoflorescenza e differenti tipi di PCR (reazioni descritte in Annesso I).

Le prove che l'OIE consiglia per la diagnostica e la movimentazione internazionale degli animali sono sierologiche e questo presenta un limite nell'individuare gli animali malati nel primo stadio della malattia, quando ancora non sono stati sviluppati anticorpi specifici e rende difficoltosa la distinzione tra animali portatori e sieropositivi.

In questo studio, sono stati selezionati 103 campioni di sangue di equini sintomatici e sono stati effettuati 8 tipi di PCR, quattro diverse per ogni tipo di piroplasma; end point, nested, real time adattata da un protocollo descritto in letteratura e un kit commerciale. Inoltre sono stati preparati strisci sanguigni e sono stati testati con i test sierologici descritti dall'OIE (ELISA e IFI). I campioni discordanti sono stati sequenziati per osservare la specificità del risultato.

Per ambidue i parassiti, le PCR più specifiche sono state le PCR real time prese dalla letteratura e sono state usate come riferimento per calcolare la sensibilità e la specificità relative (rSe, rSp); è anche stata calcolata la percentuale di accordo osservato fra le tecniche.

Tra le tecniche per diagnosticare *B.caballi*, quella che ha mostrato maggior rSe è stata lo striscio e quella che ha mostrato maggior rSp è stata l'ELISA. In quanto alle tecniche per *T. equi* la maggior rSe è stata osservata nella IFI e la maggiore rSp nello striscio. La percentuale di accordo osservato tra la real time e le altre tecniche va dal 78 al 96%. Alla vista dei risultati, si ritiene necessario aggiungere una tecnica di diagnostica molecolare alla prova sierologica per diagnosticare la piroplasmosi equina e discriminare tra sieropositivi, portatori e animali infettati con forme acute.

Contributo dell'autrice: elaborazione di tutte le PCR eccetto il kit commerciale, estrazione e purificazione del DNA per sequenziamento; il 30 % delle analisi IFI; calcoli dei valori di rSe, rSp e accordo osservato fra tecniche e redazione dello scritto.

STUDY 1

Comparison of blood smear, serological and molecular techniques for the diagnosis of equine piroplasmosis

Abstract

Babesia caballi and *Theileria equi* are the causative agents of equine piroplasmosis, a tick-borne disease affecting equids. OIE prescribed tests are all based on serological techiniques and no gold standard test has been determined so in this study a comparison of direct and indirect methods was performed in order to evaluate the different test available to diagnose equine piroplasmosis.

To this purpose, 103 blood samples were collected from symptomatic equids and four different kinds of PCR for each parasite were conducted (end point, nested, real time from litterature protocol and commercial kit). Blood smears were also examined and sera were tested by IFAT and ELISA. Sequencing on discordant results was performed. Real time protocols adapted from literarure showed the most specific PCRs. Agreement among tests and relative sensitivity (rSe) and relative specificity (rSp) were calculated for all techniques; Chi square test was used to determine statistically significant differences among PCR tests results, between direct methods (PCR and blood smear) and indirect methods (IFAT and ELISA). For *B.caballi,* the blood smear technique showed the higher rSe and the ELISA assay the higher specificity. For *T.equi* IFAT showed the highest rSe and blood smear the highest rSp. Agreement between real time PCR and the other techniques was always higher than 78% (78%-96%). Significant differences were found within direct methods and indirect methods but no among PCR test results.

Molecular tests are a very important tool in routine diagnosis because it allows to differentiate carriers from seropositive animals and to detect infected animals in early stages.

Introduction

Equine piroplasmosis is a life threatening tick-borne disease caused by *Babesia caballi* and *Theileria equi* that affects horses, mules, donkeys and zebras. The symptoms of this disease range from acute fever, anorexia and malaise, to anemia and jaundice, sudden death,

or chronic weight loss and poor exercise tolerance. Piroplasmosis is a major constraint to the international movement of equines (OIE 2011). There is no gold standard detection technique but currently, the indirect fluorescent antibody test (IFAT) and the competitive enzyme-linked immunosorbent assay (c-ELISA) are the prescribed tests recommended by the OIE for international trade. Due to the fact that antibodies last for a long period of time, up to 4 years in *Babesia* infections and lifelong in *Theileria* infections (de Waal, 1992); it is important to determine an accurate method to identify the agent such as PCR to differentiate seropositive from sick animals or diagnose the disease in the early stages of the infection when animals are still seronegative.

The first objective of this study was to identify a method to define the carrier status, in suspects and seropositive animals. A second objective was to detect infections in the early stage. Finally, we aimed to select a suitable PCR for the development of a quantitative method to assess correlation between parasitemia and the clinical phase of infection, in order to aid the practitioner in prognosis and treatment (side effects of drugs).

Materials and methods

Samples

Whole blood and EDTA samples (n=103) were collected from suspicious equids with clinical signs (case definition accepted animals showing at least one of these symptoms: fever, jaundice, and anemia).

Sera were obtained by centrifugation at 358 g for 10 minutes; then stored at –20°C and –80°C until further use.

Blood smear

Piroplasms were diagnosed microscopically in Giemsa-stained films of EDTA-anticoagulated blood. All field samples were examined at x1000 magnification, before the sample was declared free of piroplasms.

Serological tests

Indirect fluorescent antibody test (IFAT) was used for the detection of specific IgG antibodies against *T. equi* and *B. caballi* infections. Tests were carried out according to the

manufacturer's instructions (Fuller Laboratories, Fullerton, California, USA). Samples with a strong fluorescence at a dilution of 1:80 were considered to be positive.

Two commercial competitive enzyme-linked immunosorbent assays (cELISA): *Babesia equi* Antibody test kit VMRD® and *Babesia caballi* Antibody test kit VMRD® were performed according to manufacturers instructions.

PCR amplification protocols and sequencing

Four different protocols for each parasite were conducted; for *Babesia caballi* an end point PCR targeting the RAP gene (B1), a nested PCR targeting the BC48 gene (B2), a real time protocol targeting the 18 S gene (B3) and a commercial kit (B4) were carried out. For *Theileria equi* an end point PCR targeting the EMA-1 gene (T1), a nested PCR targeting the EMA-1 gene (T2), a real time protocol targeting the 18 S gene (T3) and a commercial kit (T4). Furhther information about the reactions volumes and detection limits can be found in the Appendix I.

DNA extraction from blood

DNA extraction from blood was conducted using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) following the protocol described by the manufacturers. The DNA yield was determined with a spectrophotometer (Eppendorf BioPhotometer, Eppendorf AG, Hamburg).

RAP end point PCR and BC48 nested PCR for the detection of B. caballi

The RAP end point PCR amplified a 825bp portion of RAP-1 gene from *B. caballi* that codes for the 493 amino acids Rhoptry-Associated Protein, RAP-1 is a protein complex of the merozoite related to binding and red cell invasion; the primers used (BC-RAP2F/BCRAP2R) were reported in literature (Bhoora *et al.*, 2010).

The BC48 nested PCR amplified a terminal fragment of 430bp BC48 gene from *B. caballi* that codes for the 48KDa immunodominant protein of Rhoptry Proteins Complex and the primers for PCR1 (BC48F1/BC48R31) and PCR2 (BC48F11/BC48R31) were reported in literature (Ikadai *et al.*, 1999; Battsetseg *et al*., 2001).

The amplifications were carried out in Gene Amp® PCR System 9700 (A. Biosystems) and PCR products were visualized after electrophoresis in a 1.5% Tris-Boric Acid-EDTA agarose gel and stained with GelRed 10,000X (Biotium, Hayward, CA,USA).

EMA-1 end point PCR and EMA-1 nested PCR for the detection of T.equi

The EMA-1 end point PCR amplifed a 268 bp portion of EMA-1 gene from *T.equi* that codes for the merozoite antigen 1 (EMA-1), an inmunodominant protein on the surface of the merozoite and the primers used (EMA-5/EMA-6) were reported in literature (Battsetseg *et al.*, 2002).

The EMA-1 nested PCR amplified a 102 bp segment of the EMA-1 gene of *T. equi* by the use of two sets of primers (EMAE-F/EMAE-R for PCR1 and EMAI-F/EMAI-R for PCR2) reported in literature that were designed based on the EMA-1 gene sequence (Kappmeyer *et al*., 1993; GenBank accession number L13784) (Nicolaiewsky *et al.*, 2001).

All amplifications were carried out in Gene Amp® PCR System 9700 (A. Biosystems) and PCR products were visualized after electrophoresis in a 1.5% Tris-Boric Acid-EDTA agarose gel and stained with GelRed 10,000X (Biotium, Hayward, CA,USA).

Real time PCR protocols used from literature

Real time PCR 18S rRNA *B. caballi* amplified a 95bp fragment in the V4 hypervariable region of 18S rRNA gene of *B. caballi*. Primers and probe employed (F: Bc-18SF402; R: Bc-18SR496; Probe: TaqMan MGB™ probe (FAM-MGB), Bc-18SP) were those reported in literature (Bhoora *et al.*, 2010). The real time 18S rRNA *T. equi* amplified an 81bp fragment in the V4 hypervariable region of 18S rRNA gene. Primers and probe employed (F: Be18SF; R: Be18SR) TaqMan probe (VIC-TAMRA, Be 18SP) were those reported in literature (Kim *et al.*, 2008). For both, a real Time PCR, TaqMan® Universal PCR Master Mix kit (A. Biosystems, Foster City, CA, USA) was used. Positive controls were constituted by plasmid vectors pCRII®-TOPO TA Cloning® Invitrogen, Carlsbad, CA, USA) in which the targets of the real time PCR *T. equi* and *B. caballi* were cloned. In each run of the quantitative real time PCR, 2 quantified internal control, developed as described further on, were included for the estimation of the protozoa DNA copies/ml of the blood samples. All data were analysed using the 7900 HT Sequence Detection Systems SDS software package (A. Biosystems).

Construction of the internal standard for qReal Time PCRs and limit detections

The V4 hypervariable regions of the 18S rRNA related to *B.caballi* and *T. equi* genes were amplified by blood samples certified positive for *B.caballi* and *T.equi* by CRABaRT (Centro

di Referenza Nazionale per Anaplasma, Babesia, Rickettsia e Theileria). Two sets of primers (RLB-F/RLB-R and RLB-F2/RLB-R2) specific for *Theileria* and *Babesia* species were used (Kouam *et al.*, 2010; Nagore *et al*., 2004). The number of V4 molecules from *B.caballi* and V4 molecules from *T. equi* was calculated on the basis of the DNA concentration, measured using a spectrophotometer. The LOD of the tests were established using the V4 regions of *B. caballi* and of *T. equi*. Aliquots of log10 dilutions of V4 regions were prepared to cover a range of dilutions between 4.35 x 10^{12} to 0.60 copy/µl for *B. caballi* and 3.96 x 10^{12} to 0.96 copy/µl for *T. equi* and were conserved at -80°C in volume of 5µl and used only once. The LOD test was carried out on three independent occasions and the threshold cycle (Ct) values were used for the construction of a standard curve. The threshold limit was set in the exponential phase of the reactions and Ct values greater than 49 were considered as negative. The standard curves were obtained from the linear regression line through the data points on a plot of Ct versus the logarithm of the standard concentration. The amount of DNA from the unknown samples was determined by interpolation of the Ct value obtained for each sample and corrected to DNA protozoan copies/ml of blood. The Ct value of each sample was represented by the mean obtained from the analyses of the sample in double replicate.

The limit of detection for qReal Time 18S rRNA *B. caballi* was 1.14 x 10^{-4} parasited erythrocytes (PE) equating to a quantification cycle (Cq) of 40.86 and the limit of detection for qReal Time 18S rRNA *T. equi* was 1.9 x 10^{-4} (PE) equating to a quantification cycle (Cq) of 45 .

Commercial real time for T. equi *and* B. caballi

Two commercial kits were employed (Genesig® Standard kit (Primerdesign™ Ltd, Southampton, United Kingdom). The real time PCR for *T. equi* amplifies a ~120bp fragment in the *T. equi* merozoite antigen 1 (EMA-1) gene; while real time PCR for *B. caballi* amplifies a ~100bp fragment in the 18S ribosomal RNA gene. Master mix were constituted according to the manufacturer's instructions. Data were analysed using the 7900 HT Sequence Detection Systems SDS software package (A. Biosystems).

Sequencing

On samples showing discordance results sequencing was performed to assess the specificity.

PCR products were visualized after electrophoresis in a 1.5% Tris-Boric Acid-EDTA agarose gel and stained with GelRed 10,000X (Biotium, Hayward, CA,USA) and all the products were recovered from agarose gel using the QIAquick® PCR Purification kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions and sequenced using the PCR primers of second PCR with the BigDye Terminator Cycle Sequencing Ready Reaction kit, version 3.1 (PerkinElmer, A. Biosystems, Foster City, CA, USA) in an automated sequencer (3500 Genetic Analyzer, A. Biosystems, Foster City, CA, USA). The nucleotide sequences obtained were analysed using the Genetic Analyzer Sequencing v5.4 (A. Biosystems, Foster City, CA, USA). Sequence homology was performed using the Basic Local Alignment Search Tool (BLAST) by comparing to sequences of equine piroplasms in NCBI GenBank (http://www.ncbi.nlm.nih.gov).

Sensitivity and specificity of the techniques

Agreement among PCRS was estimated. The assessment of relative sensibility (rSe) and relative specificity (rSp) were calculated using the PCR detecting the greatest number of confirmed positives. Agreement between all techniques was also estimated for each protozoan. Chi square test was used to evaluate the results differences within direct and indirect methods and among PCR assays (Thrurfield, 2007).

Results and discussion

All results related to agreement, relative specificity and relative sensibility are described in the tables 1 and 2.

From the 103 samples, ten resulted positive to *B.caballi* PCR; 4 were positive to the end point PCR (B1), 8 positive to the nested PCR (B2), 4 positive to the real time PCR literature protocol (B3) and 2 positive to the commercial test (B4). However only samples detected by real time PCR were confirmed by sequencing. Agreement among the 4 techniques was 91.3% (94 concordant results to 4 tests, 4 concordant results to 3 tests and 5 concordant results to 2 tests).

Table 1: General agreement of PCR

	Number of PCRs in agreement			General agreement
	Four	Three	Two	
B. caballi	94	4	5	91.30%
T. equi	93	6	4	90.30%

When evaluating *Babesia caballi* PCRs we observe that B3 has the highest sensibility, specificity and primer efficiency. The target length is short, being the most appropriate. Taqman MGB makes up for high mutation frequency and its short amplicon avoids problem related to target degradation (often in *B. caballi*).

From the 103 samples 36 resulted positive to *T.equi*; 29 were positive to the end point (T1), 29 positive to the nested (T2), 35 positive to the real time PCR literature protocol and 27 positive to the Genesig. Agreement among the 4 PCRs was 90.3%. (93 concordant results to 4 tests, 6 concordant results to 3 tests and 4 concordant results to 2 tests). In the 35 positives detected by real time (T3) all the other PCRs are included except one positive from nested PCR that was not confirmed by sequencing.

The T3 showed the highest sensibility, specificity and primer efficiency. The target length was the shortest, but the most appropriate. Taqman probe was designed in a high conserved region.

Table 2: Positive results related to molecular methods.

	Positive results by method			
	End-point	Nested	Real Time	Kit
B. caballi	4	8	4	2
T. equi	29	29	35	27

B3 and T3 detected the greatest number of confirmed positives (4 and 35) and were considered the best PCRs probably due to their primer efficiency and their short amplicons. They both were chosen to evaluate rSe and rSp. Sensibility in *T.equi* PCRs ranged from 77% to 80% and specificity from 98% to 100%. For *B.caballi* rSe ranged from 25% to 50% and rSp from 93.94% to 100%, the lower rSe of B1 and B2 could be due to the higher mutation frequency or

degradation of their long targets, but recruitment of a major number of positives is necessary to verify this result (Bhoora *et al.,* 2010).

When setting a PCR the ideal target characteristics are short length to counteract different issues such as poor extraction efficiency, low parasitemia (1% for *B. caballi* as described by Hanafusa *et al.,* 1998) or DNA degradation; designed based on constitutive genes and high conserved regions.

The results obtained from T3 and B3 were compared to serological and blood smears results and rSe, rSp and agreement were also calculated. Table 3.

For *B. caballi* all ELISA samples were negative and 3 IFAT samples presented discordant results (IFAT negative and PCR positive) and for *T.equi* 17 PCR positive samples were ELISA negative and 4 resulted negative to IFAT being PCR positive, these discordances could be due to acute forms of piroplasmosis. Within the negative PCR samples 9 were positive in IFAT to *B. caballi* and 10 to *T.equi* and 4 samples were positive to *T.equi* ELISA, so these seropositive animals although showed symptoms were not carriers.

In positive PCR animals for *B. caballi* 2 samples were not positive to the blood smear test and for *Theileria* 17 presented the same discordance, this fact could be probably due to the low loads of parasites detectable by PCR but not by microcopy observation. In some cases, negative animals by PCR were positive to blood smear examinations (16 for *Babesia* and 2 for *Theileria*), thorough analyses showed that in some cases a cross diagnosis happened as the blood smear report didn't discriminate between species.

For *B.caballi* diagnostic techniques the rSe ranged from 0% (ELISA) to 50% (blood smear) and the rSp from 82% (IFAT) to 100% (ELISA). The overall agreement ranged from 81% (blood smear) to 96% (ELISA. The agreement between serological methods was 90.3%.

For *T.equi* tests the rSe ranged from 51% (ELISA) to 88% (IFAT) and the rSp from 82% (IFAT) to 96% (blood smear). The overall agreement ranged from 78% (ELISA) to 84% (IFAT). The agreement between serological methods was 76.7%.

Table 3: Results of rSe, rSp and agreement within techniques.

	B3		
	rSe	**rSp**	**Agreement**
B1	25.00	96.97	94.17
B2	50.00	93.94	92.23
B4	50.00	100.00	98.06
	T3		
	rSe	**rSp**	**Agreement**
T1	80.00	98.53	92.23
T2	82.86	100.00	94.17
T4	77.14	100.00	92.23
	B3		
	rSe	**rSp**	**Agreement**
ELISA BC	0.00	100.00	96.12
IFAT BC	25.00	90.91	88.35
Blood smear	50.00	82.61	81.25
	T3		
	rSe	**rSp**	**Agreement**
ELISA TE	51.43	96.25	78.64
IFAT TE	88.57	82.35	84.47
Blood smear	51.61	96.92	82.29
	IFAT BC		
	rSe	**rSp**	**Agreement**
ELISA BC	0.00	100.00	90.29
	IFAT TE		
	rSe	**rSp**	**Agreement**
ELISA TE	48.84	96.67	76.70

Significant differences were found within direct and indirect methods but no among PCR test results for both parasites (Table 4), these results partially agree with those from other authors (Mahmoud *et al*., 2016).

Table 4: Chi square p values (significant p<0.05)

	PCR assays	**Direct methods**	**Indirect methods**
B. caballi	0.167	0.0015	0.0016
T. equi	0.638	0.03	0.004

IFAT technique is more sensitive than other techniques used before such as complement fixation test in cronic infections (Brüning. 1996). However, it presents some limits due to the presence of cross reactions (so that is why is needed to perform dilutions to identify the pathogen in these cases) and interpretation sometimes depends on technical expertise.

ELISA is an easy, convenient and quick test and it has been recognised as the most sensitive test to identify cronic and unapparent carriers (Knowles *et al.*, 1992), however it is based on the recognition of one epitope that it could be not very conserved among isolates (Bhoora *et al*., 2010; Rapoport *et al*., 2014), in fact for *B.caballi* no sample was positive in our assay although animals were detected using other methods.

Blood smear is still a reliable tool but good skills to exam the samples are needed, the limit to this test is also fhe low parasitemia that piroplasmosis shows even in acute phases of infection (Wise *et al.,* 2014).

Our results showed fair rSe for almost all methods, and high rSp for all tests ranging from 82% to 100%; for *B. caballi* the most sensitive was the blood smear test and the most specific was the ELISA assay. However, for *T.equi* the IFAT was the most sensitive and the blood smear test the most specific.

Conclusions

Discordant results are a fact and not always related to the sensitivity limit of the detection assay. A correct interpretation of data is essencial to properly diagnose equine piroplasmosis.

A real time protocol either for *Babesia* (B3) and *Theileria* (T3) showed to be the most suitable tests to develop a quantitative method to assess correlation between parasitemia and the clinical phase of infection. Blood smear and serological tests showed high rSp and fair

values of rSe so our results showed no technique can be reccomended or excluded for diagnostics, but should be complemented with a molecular assay.

Seropositive animals in endemic regions should be confirmed by PCR to avoid unnecesary treatments. Symtomatic seronegative should also be checked to detect the infection in early stages.

Moreover, molecular tests are useful in routine diagnosis, aiding practitiones to decide or verify treatment and prognosis. Together with serological tests, they are also helpful for the control of animals for international movement.

Author contribution

Elaboration of all PCRs except commercial kits, extraction and purification of DNA for sequencing; some of the IFAT analysis; Calculations of rSe, rSp and agreement. Manuscript writting.

Acknowledgements

This study was developed with funds from the IZSLT 16/11 project (provided by the Italian Ministry of Health). Thanks to doctor Rosone and Scarpulla for the technical support.

Conflict of interest

The author declare no conflict of interests.

References

Battsetseg, B.; Xuan, X.; Ikadai, H.; Bautista, J. L. R.; Byambaa, B.; Boldbaatar, D.; Battur, B.; Battsetseg, G.; Batsukh, Z.; Igarashi, I.; Nagasawa, H.; Mikami, T.; Fujisaki, K. Detection of *Babesia caballi* and *Babesia equi* in *Dermacentor nuttalli* adult ticks. *Int. J. Parasitol.* **2001**, *31*, 384–386.3.

Battsetseg, B.; Lucero, S.; Xuan, X.; Claveria, F. G.; Inoue, N.; Alhassan, A.; Kanno, T.; Igarashi, I.; Nagasawa, H.; Mikami, T.; Fujisaki, K. Detection of natural infection of Boophilus microplus with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. *Vet. Parasitol.* **2002**, *107*, 351–357.2.

Bhoora, R.; Quan, M.; Franssen, L.; Butler, C. M.; Van der Kolk, J. H.; Guthrie, A. J.; Zweygarth, E.;

Jongejan, F.; Collins, N. E. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. *Vet. Parasitol.* **2010**, *168*, 201–211.4.

Bhoora, R.; Quan, M.; Zweygarth, E.; Guthrie, A. J.; Prinsloo, S. a.; Collins, N. E. Sequence heterogeneity in the gene encoding the rhoptry-associated protein-1 (RAP-1) of *Babesia caballi* isolates from South Africa. *Vet. Parasitol.* **2010**, *169*, 279–288.5.

Brüning, A. Equine piroplasmosis an update on diagnosis, treatment and prevention. *Br. Vet. J.* **1996**, *152*, 139–151.6.

de Waal, D. T. Equine piroplasmosis: a review. *Br. Vet. J.* **1992**, *148*, 6–14.

Hanafusa, Y.; Cho, K. O.; Kanemaru, T.; Wada, R.; Sugimoto, C.; Onuma, M. Pathogenesis of *Babesia caballi* infection in experimental horses. *J. Vet. Med. Sci.* **1998**, *60*, 1127–32.7.

Ikadai, H.; Xuan, X.; Igarashi, I.; Tanaka, S.; Kanemaru, T.; Nagasawa, H.; Fujisaki, K.; Suzuki, N.; Mikami, T. Cloning and expression of a 48-kilodalton *Babesia caballi* merozoite rhoptry protein and potential use of the recombinant antigen in an enzyme- linked immunosorbent assay. *J. Clin. Microbiol.* **1999**, *37*, 3475–3480.8.

Kappmeyer, L. S.; Perryman, L. E.; Knowles, D. P. A *Babesia equi* gene encodes a surface protein with homology to *Theileria* species. *Mol. Biochem. Parasitol.* **1993**, *62*, 121–124.10.

Kim, C. M.; Blanco, L. B. C.; Alhassan, A.; Iseki, H.; Yokoyama, N.; Xuan, X.; Igarashi, I. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. *Vet. Parasitol.* **2008**, *151*, 158–163.12.

Knowles, D. P.; Kappmeyer, L. S.; Stiller, D.; Hennager, S. G.; Perryman, L. E. Antibody to a recombinant merozoite protein epitope identifies horses infected with *Babesia equi*. *J. Clin. Microbiol.* **1992**, *30*, 3122–3126.13.

Mahmoud, M. S.; El-Ezz, N. T. A.; Abdel-Shafy, S.; Nassar, S. A.; El Namaky, A. H.; Khalil, W. K. B.; Knowles, D.; Kappmeyer, L.; Silva, M. G.; Suarez, C. E. Assessment of *Theileria equi* and *Babesia caballi* infections in equine populations in Egypt by molecular, serological and hematological approaches. *Parasit. Vectors* **2016**, *9*, 260.14.

Nagore, D.; García-Sanmartín, J.; García-Pérez, A. L.; Juste, R. a.; Hurtado, A. Detection and

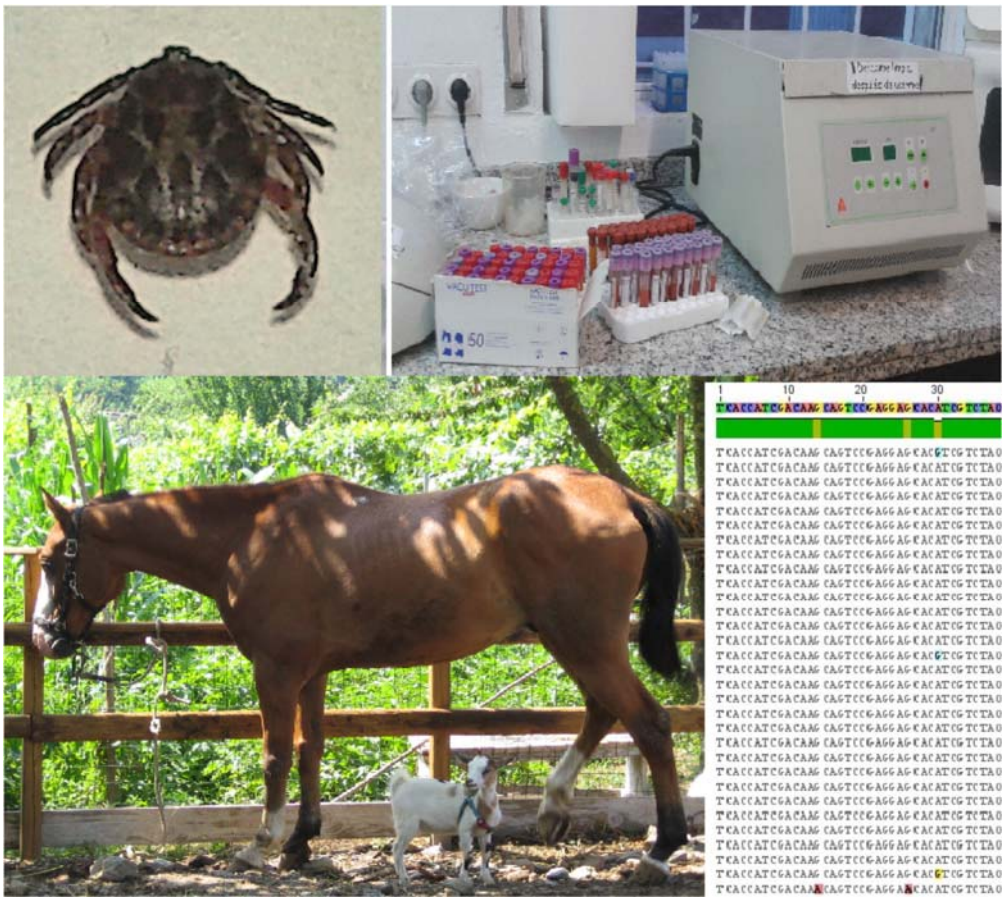
identification of equine *Theileria* and *Babesia* species by reverse line blotting: Epidemiological survey and phylogenetic analysis. *Vet. Parasitol.* **2004**, *123*, 41–54.15.

Nicolaiewsky, T. B.; Richter, M. F.; Lunge, V. R.; Cunha, C. W.; Delagostin, O.; Ikuta, N.; Fonseca, A. S.; Da Silva, S. S.; Ozaki, L. S. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. *Vet. Parasitol.* **2001**, *101*, 9–21.16.

Rapoport, A.; Aharonson-Raz, K.; Berlin, D.; Tal, S.; Gottlieb, Y.; Klement, E.; Steinman, A. Molecular characterization of the *Babesia caballi* RAP-1 gene and epidemiological survey in horses in Israel. *Infect. Genet. Evol.* **2014**, *23*, 115–120.

Thrusfield, M. Veterinary Epidemiology, 3rd Edition. **2007.** Wiley-Blackwell, USA, 624 pp.

Wise, L. N.; Pelzel-McCluskey, A. M.; Knowles, D. P. Equine Piroplasmosis. *Vet. Clin. North Am. Equine Pract.* **2014,** *30*, 677–693.



CAPÍTULO 2: EPIDEMIOLOGÍA Y FILOGÉNESIS

CHAPTER 2: EPIDEMIOLOGY AND PHYLOGENETICS

CAPITOLO 2: EPIDEMIOLOGIA E FILOGENESI

RESUMEN CAPÍTULO 2

En este capítulo se presentan los resultados de dos estudios de prevalencia, uno en España y otro en Italia, un tercer estudio sobre análisis filogenético de los genotipos encontrados y un estudio que evidencia de la transmisión vertical de estos parásitos.

El primer estudio llevado a cabo en la zona central de la Península Ibérica analiza la seroprevalencia de 179 équidos asintomáticos (139 caballos y 40 asnos) usando kits comerciales de ELISA; se evaluaron estadísticamente (análisis univariado) factores de riesgo como la especie, edad, género, raza, color de la capa, aptitud, uso de antiparasitarios, acceso a pasto, presencia de garrapatas, introducción reciente en la explotación, presencia de otras especies animales, altitud, cobertura terrestre, zona climática y tipo de suelo.

La prevalencia total para *T. equi* fue 22,3% (40/179) y para *B. caballi fue* 2,8% (5/179). Todos los factores de riesgo asociados a *T.equi* fueron significativos, excepto la especie y la introducción reciente. En el caso de *B.caballi* solo resultó significativo el color de la capa.

En el segundo estudio, realizado en la zona centro-sur de Italia se analizaron muestras de 177 burros y 673 caballos asintomáticos mediante ELISA y los caballos seropositivos (263), junto a todos los asnos, se testaron usando PCR para determinar la presencia de portadores sanos. Se seleccionaron diversos parámetros para evaluar los factores de riesgo similares al estudio 1. En los burros debido a la falta de datos solo se estudiaron género, raza, edad y localización de la granja.

En caballos la prevalencia total de *T.equi* fue del 39,8% (268/673) y de *B. caballi* 8,9% (60/673); las muestras serológicamente positivas se testaron también con PCR y el 70,3% (185/263) resultó positiva a *T. equi y el* 10,3% (27/263) a *B. caballi.* Los análisis estadísticos confirmaron que los factores de riesgo asociados a la seropositividad en *T.equi* fueron la edad, el género, la raza, el acceso al pasto, la cobertura terrestre, la localización de la provincia, y el tipo de suelo. En la seroprevalencia de *B.caballi* resultaron los mismos factores, excepto la zona climática en lugar de la edad. Los factores asociados a la positividad en PCR fueron edad, y tipo de suelo.

En burros la seroprevalencia en ELISA fue del 54,8% (97/177); no se observaron muestras positivas para *Babesia.* En PCR se observó una prevalencia del 59,8% (101/169) y del

5,9% (10/169) en *T. equi* y *B.caballi* respectivamente. Los factores de riesgo asociados a *T. equi* fueron el género en el análisis serológico y la zona en la PCR.

En una zona en la que muchos caballos del estudio habían resultado seropositivos y portadores se recogieron un total de 26 garrapatas con la técnica del arrastre de manta, todas ellas de la especie *Dermacentor marginatus,* 15 de estos vectores fueron positivos a *T.equi* en PCR, ninguna fue positiva a *B. caballi.*

En el tercer estudio, se seleccionaron 100 muestras de animales sintomáticos y asintomáticos (muchos del segundo estudio) en base a dos criterios, discordancia entre prueba serológica y PCR o fuerte positividad en PCR en tiempo real. Se llevó a cabo una PCR anidada que amplifica la región V4 del 18rDNA ("PCR V4") capaz de tipificar diferentes piroplasmas. También se testaron con otra PCR de punto final que amplifica el gen del merozoíto de *T.equi* (EMA-1). Las garrapatas recogidas en el segundo estudio también se testaron con esta PCR ("PCR EMA"). Las secuencias mostraron un mínimo de "consulta de cobertura" y de homología con otras secuencias de GenBank del 98%.

El análisis filogenético de los amplificados obtenidos con la "PCR V4" incluía distancia y homología y confirmó la presencia de tres grupos en ambos parásitos. Asimismo el análisis filogenético de los productos de la "PCR EMA" también concluyó con la presencia de tres grupos en *T.equi.* Se estudiaron las diferencias entre grupos y se seleccionaron determinadas secuencias para la construcción de los árboles filogenéticos.

También se evaluaron usando Chi cuadrado o test de Fisher las relaciones entre los grupos filogenéticos y algunas variables como presencia de sintomatología, resultados serológicos discordantes, coinfección parasitaria de *Babesia* y *Theileria* y baja o ausente positividad a *T.equi* en "PCR EMA" (secuencias de *T.equi* obtenidas con la "PCR V4"). En el caso de *T. equi* resultaron significativas las relaciones entre presencia de síntomas, discordancia entre serología y PCR y entre PCRs "EMA" y "V4". En el análisis *Babesia* no hubo variables significativas. Los grupos definidos usando la "PCR V4" y la "PCR EMA" no están relacionados. No se evidencia relación entre el origen de los grupos y la distribución geográfica.

En el cuarto estudio se analizan con diferentes técnicas (serológicas, moleculares y microscópicas) muestras de yeguas sospechosas de padecer piroplasmosis y sus abortos o

material fetal, se secuenciaron los productos de las PCR y se determinó el paso transplacentario de ambas especies de piroplasmas (*T. equi* y *B.caballi*).

Los resultados obtenidos en los estudios de prevalencia confirman que las zonas centrales de España e Italia son zonas endémicas, y que se necesita mejorar las medidas de control de vectores, así como identificar los portadores para evitar la transmisión. En el tercer estudio se han obtenido resultados interesantes sobre la patogenicidad de las distintas variantes, pero se necesita profundizar en esta materia para deducir la correlación entre grupos genéticos y los patrones serológicos y clínicos. El cuarto estudio demuestra la importancia de la transmisión intrauterina y es la primera vez que se describe paso transplacentario de *B.caballi* en Europa.

Contribución de la autora: recogida de muestras de équidos (70%) y de garrapatas, elaboración de tests ELISA e IFI, selección de los factores de riesgo incluidos en el estudio, realización de las PCR, extracción de ADN, purificación, alineamiento de las secuencias, examen parcial de las mismas, construcción de los árboles filogenéticos, redacción de los textos.

RIASSUNTO CAPITOLO 2

In questo capitolo si presentano i risultati di due studi di prevalenza, un terzo studio riguardante l'analisi filogenetica dei genotipi riscontrati e sulla loro patogenicità e un quarto studio che evidenza la trasmissione trasplacentare di questi parassiti.

Il primo esperimento svolto nella zona centrale della Penisola Iberica, analizza la sieroprevalenza di 179 equini asintomatici (139 cavalli e 40 asini) usando kit commerciali di ELISA. Sono stati analizzati statisticamente (analisi univariate) fattori di rischio come la specie, età, sesso, razza, colore del mantello, attitudine, uso di antiparassitari, accesso al pascolo, presenza di zecche, introduzione recente nell'allevamento, presenza di altre specie di animali, altitudine, copertura terrestre, zona climatica e tipo di suolo.

La prevalenza totale di *T. equi* era del 22,3% (40/179) e per *B. caballi* 2,8% (5/179). Tutti i fattori di rischio associati a *T.equi* risultarono significativi eccetto la specie e l'introduzione recente. Nel caso di *B.caballi* soltanto risultò significativo il fattore colore del mantello.

Nel secondo esperimento, svolto nella zona centro-sud d'Italia sono stati analizzati i campioni di 177 asini e 673 cavalli asintomatici mediante ELISA e i cavalli sieropositivi (263), insieme a tutti gli asini, si testarono usando PCR per determinare la presenza di portatori sani. Sono stati selezionati diversi parametri per valutare i fattori di rischio associati all'infezione nei cavalli come il genere, l'età, razza, uso di antiparassitari, acceso al pascolo, altitudine, copertura terrestre, zona climatica, tipo di suolo e localizzazione della provincia. Negli asini, dovuto alla mancanza di dati, sono stati studiati solo sesso, razza, età e localizzazione dell'allevamento. Si è proceduto a fare una analisi di regressione univariata e multipla per stabilire l'influenza dei diversi fattori.

Nei cavalli la prevalenza totale di *T. equi* fu del 39,8% (268/673) e di *B. caballi* 8,9% (60/673); i campioni sierologicamente positivi si testarono inoltre con PCR e il 70,3% (185/263) risultó positivo a *T. equi* e il 10,3% (27/263) a *B. caballi*. Le analisi statistiche confermano che i fattori di rischio associati alla sieropositività in *T. equi* furono l'età, il sesso, la razza, l'accesso al pascolo, la copertura terreste, la localizzazione della provincia, e il tipo di suolo. Nella sieroprevalenza di *B.caballi* risultarono gli stessi fattori, eccetto la zona climatica al posto dell'età; i fattori associati alla positività nella PCR furono età e tipo di suolo.

Negli asini la sieroprevalenza nell'ELISA risultò del 54,8% (97/177); non sono stati osservati campioni positivi per *Babesia*. Nella PCR è stata osservata una prevalenza del 59,8% (101/169) e del 5,9% (10/169) rispettivamente in *T. equi* e *B.caballi*. I fattori di rischio associati a *T. equi* furono il sesso nell'analisi sierologica e la zona nell'analisi della PCR.

In un'area geografica dove molti cavalli dello studio sono risultati sieropositivi e portatori, vi sono state raccolte un totale di 26 zecche con la tecnica del dragging, tutte le zecche appartenevano alla specie *Dermacentor marginatus*, 15 di questi vettori risultarono positivi a *T.equi* in PCR, ma nessuna risultò positiva a *B.caballi*.

Nel terzo esperimento, sono stati selezionati 100 campioni di animali sintomatici e asintomatici (molti del secondo studio) in base a due criteri, discordanza tra prova sierologica e PCR o forte positività in real time PCR. Questi campioni si testarono con una PCR nested che amplifica la regione V4 del 18R DNA (PCR V4) capace di tipificare diversi piroplasmi. Inoltre sono stati testati con un'altra PCR end point che amplifica il gene del merozoito di *T.equi* (EMA1). Le zecche raccolte nel secondo studio sono testate anche con questa PCR (PCR EMA). Le sequenze mostrarono un minimo di "query coverage" e di omologia con altre sequenze di GenBank del 98%.

L'analisi filogenetica degli amplificati ottenuti con la PCR V4 includeva distanza e omologia e confermò la presenza di tre gruppi per entrambi i parassiti. Così come l'analisi filogenetica del prodotto della "PCR EMA" si concluse con la presenza di tre gruppi di *T.equi*. Sono state studiate le differenze tra i gruppi e sono state selezionate determinate sequenze per la costruzione degli alberi filogenetici.

Inoltre sono state valutate usando la Chi quadrato o test di Fisher le relazioni fra i gruppi filogenetici ottenuti con la "PCR V4" e alcune variabili come presenza di sintomatologia, risultati sierologici discordanti, coinfezione parassitaria di *Babesia* e *Theileria*, bassa o assente positività a *T.equi* in PCR EMA (sequenze di *T.equi*). Nel caso di *T.equi* risultarono significative le relazioni fra presenza di sintomi, discordanza fra la sierologia e PCR e fra PCR "EMA" e "V4". Nell'analisi di *Babesia* non si trovarono variabili significative. I gruppi definiti usando la "PCR V4" e la "PCR EMA" non sono relazionati.Neanche sono state evidenziate relazioni fra l'origine del gruppo e la distribuzione geografica.

Nel quarto studio sono stati analizzati con diverse tecniche (sierologia, molecolare e microscopia) campioni di cavalle sospette di essere affette da piroplasmosi e gli aborti o i materiali fetali di esse; sono stati sequenziati i prodotti della PCR ed è stato determinato il passaggio transplacentare di entrambe le specie di piroplasma (*T. equi* e *B.caballi*).

I risultati ottenuti nello studio di prevalenza confermano che le zone centrali di Spagna e Italia sono zone endemiche, e che vi risulta necessario impiantare misure di controllo dei vettori, così come identificare i portatori per evitare la trasmissione. Nel terzo esperimento sono stati ottenuti risultati interessanti sulla patogenicità delle diverse varianti, ma bisogna approfondire in questa materia per dedurre la correlazione fra gruppo genetico e pattern sierologico e clinico. Il quarto studio mostra l'importanza della trasmissione intrauterina ed è la prima volta che si conferma questa via per *B.caballi*.

Contributo dell'autrice: raccolta di campioni di equini (70%) e zecche, elaborazione di test ELISA ed IFI, selezione dei fattori di rischio inclusi nello studio, esecuzione delle PCR, estrazione di DNA, purificazione, allineamento delle sequenze, esame parziale delle stesse, costruzione dell'albero filogenetico, redazione dei testi.

STUDY 2.1

Serological detection of *Theileria equi* and *Babesia caballi* infections in horses and donkeys in areas of Central Spain and risk factors associated.

Abstract

Serum samples were collected from 179 animals (139 horses and 40 donkeys) in Central Spain and examined by competitive-inhibition ELISA (cELISA) to evaluate seroprevalence for *B. caballi* and *T. equi*. The following parameters were chosen to evaluate the risk factors related to equine piroplasmosis: equid species, gender, age, breed, colour coat, activity, external parasite treatments, access to pasture, presence of ticks, introduction of new equids, presence of other species, altitude of the farm, land cover, climatic zone and soil type. A linear univariate analysis was performed.

The overall seroprevalence for *T. equi* was 22.3% (40/179) and for *B. caballi* was 2.8% (5/179). All the studied risk factors associated to *T. equi* seroprevalence were significant except species and introduction of new animals. For *B. caballi* colour coat was found to be the only significant factor.

These results confirm the status of Spain as an endemic country and indicate the need to improve control programmes to identify carriers and to prevent the spread of the disease.

Introduction

Equine piroplasmosis (EP) is a tick-borne disease caused by *Babesia caballi* and *Theileria equi* that affects horses, mules, donkeys and zebras. Both parasites are transmitted by ticks of genera *Dermacentor, Rhipicehalus and Hyalomma.* The disease is globally distributed and causes subclinical and clinical infections, and death. Antibodies are long-lasting, 4 years for *Babesia,* lifelong for *Theileria* (de Waal, 1992). Diagnostics can be performed by stained blood identification, serological tests such as complement fixation test, indirect fluorescent antibody test (IFAT), ELISA or PCR methods. Equine piroplasmosis is responsible of important economic losses in the equine sector being a major constraint to the international movement of equines (OIE 2011).

To date, information on the epidemiology of equine piroplasmosis in Central Spain is limited, in previous studies conducted in this Animal Health Department, prevalences determined by IFAT for *B caballi* and *T. equi* were 13 % and 37.6 % respectively (Olmeda *et al.*, 2000) and around 12 % for *B. caballi* ranging from 20 to 33% for *T. equi* (Olmeda *et al.*, 2001). In both studies over 10% of the samples presented coinfection. In other studies performed in several Spanish regions *T.equi* seroprevalences ranged from 40% detected by IFAT (Camacho *et al.*, 2005) to 52.5% (Habela *et al*., 2000) to 56.1% detected by ELISA (García Bocanegra *et al*., 2012), *B. caballi* seroprevalence detected by IFAT by the same authors were over the 20% although García-Bocanegra *et al.* (2012) determined a lower seroprevalence (13.2%) by ELISA. In Portugal, Ribeiro *et al.* (2013) reported lower prevalences determined by ELISA, 17.9% and 11.1 % for *Theileria* and *Babesia* respectively. Several studies with discordant data about donkey piroplasmosis have been conducted in different countries, i.e. in Brazil by Machado *et al.* (2012), Ethiopia by Mekibib *et al.* (2010), Tefera *et al.* (2011), and Gizachew *et al.* (2012), in Kenya by Oduori *et al.* (2015); in China by Chahan *et al.* (2006), in Eastern Turkey by Balkaya *et al.* (2010). In Europe, some data about piroplasmosis seroprevalence in donkeys within a horse study have been provided by García-Bocanegra e*t al.* (2012) in Southern Spain.

The aim of this study was to determine seroprevalences of both parasites in central areas of Spain and to identify associated risk factors.

Materials and methods

Sampling and risk factors studied

This study involved the equine population in Central Spain. Equidae sera (n=179) from asymptomatic animals in different epidemiological situations (41 rural area equids, 47 city area Police horses, 66 sport horses and 25 breeding facility mares) were tested.

Blood samples were collected from jugular venipuncture; sera were obtained by centrifugation at 358 g for 10 minutes; then stored at –20°C until used.

To evaluate risk factors related to EP data regarding the following parameters were registered: equid species (donkey/horse), sex (male /gelding/female), age (young <= 6; adult 7-12; senior > 12 years old,), colour coat (light/dark), breed was categorized in three groups

(foreign breeds, autochthonous breeds, and mixed breed), activity (recreation/sport/breeding/police horse), external parasite treatment and vaccines (regularly/no/occasionaly), access to pasture (yes/no), presence of ticks (yes/no) new introduction in the farm in the last 6 months (yes/no), and cohabitation with other species (yes/no). In order to add more variables to the risk factors' evaluation file , geographic locations of the farms were determined using a GPS system that measured altitude (250-600 meters;> 600meters); land cover (crops 50-75% and mixed, with no dominant land cover); climate zone based on length of growing period (LGP) the period (in days) during a year when precipitation exceeds half the potential evapotranspiration (humid LGP 270-365 days, sub-humid LGP 180-269 days; moist-semiarid LGP 120-179 days), and soil type (eutric cambisol with a base saturation of 50% or more; dystrict cambisol with a base saturation of less than 50%; xerosol a kind of sub-arid soil and planosol characterized by a suburface layer of clay accumulation).

Land cover, climate and soil information were obtained using the geographic coordinates in the interactive maps available on FAO site. http://www.fao.org/geonetwork/srv/en/main.home.

Some variables such as climatic zone (all equids were present in the sub-humid zone) and deworming and vaccines (all animals were regularly dewormed and vaccinated) were not included in the model.

Serological tests

Two commercial competitive enzyme-linked immunosorbent assays (cELISA) were employed: *Babesia equi* Antibody test kit VMRD® and *Babesia caballi* Antibody test kit VMRD®; according to manufacturers instructions.

Statistical analysis

Serological prevalence and their 95% confidence intervals (95% CI) were calculated) in terms of area of study and for each risk factor investigated, as shown in the literature (Thursfield, 2007). Relations between explanatory variables and piroplasm seropositivity were evaluated by Chi square or Fisher's exact test. A p value <0.05 was considered statistically significant. SAS 9.4 software for Windows was used for all statistical analyses.

Results

Serological prevalence and correspondent IC 95% for each variable are shown in Table 1.

T. equi

The overall seroprevalence for *T. equi* was 22.3% (40/179, CI 95%: 17-29%).

The following parameters resulted significant ($p<0.05$) in the univariate model: gender, age, breed, colour coat, activity, external parasite treatment, access to pasture, presence of ticks, presence of other species, altitude of the farm, land cover, and soil type.

There were differences among geldings, males and females prevalences, being the first significantly higher ($p<0.001$).

Age was a significant variable, the older the higher the seroprevalences ($p<0.0001$).

Significant differences ($p<0.04$) were found regarding breed among horses: the seroprevalence in the mixed breed group was higher than in the pure breed groups; between the groups of pure breeds the native group showed a higher prevalence than the foreign one. Among donkeys no significant differences were found related to breed.

Colour coat was also a significant variable ($p=0,004$), light coated animals showed higher prevalence than dark ones. Differences related to the activity were also significant ($p<0.0001$) with higher highest prevalences were found in Police horses, followed by breeding mares and recreation equids; the sport horses showed the lowest prevalence. Differences in the external parasite treatments frequency were found ($p<0.0001$). Contact with other species was also a significant parameter ($p<0.0001$); animals in constant contact with ruminants and dogs showed a higher prevalence than those sporadically meeting other species.

Higher prevalence in horses with access to pasture was also significant ($p=0.001$). Tick presence on the animals ($p=0.018$) was also a confirmed risk factor. However, no statistical difference related to new introduction of animals in the farms was detected.

Altitude was a significant factor ($p<0.0001$): the highest prevalence was found in the group ranged from 250-600m ($p<0.01$).

Regarding the soil parameter (p<0.0001), the planosol group showed the highest prevalence, followed by the xerosol and the cambisols groups.

Land cover was also a significant variable (p=0.005) the animals from mixed areas showed higher prevalence values than the animals from crops areas.

B. caballi

The overall seroprevalence was 2.8% (5/179; CI 95%: 1-6%).

Many prevalence differences within categories were similar to the factors described for *T. equi* (all data are reported in Table 1) however the only significant parameter (p<0.036) in the model was colour coat; light colour coat animals presented higher prevalence than those of darker colour coat.

Discussion

In this study 179 samples from asymptomatic equids were examined to determine the seroprevalence for EP.

Prevalence for *T. equi* was 22.3%, this result is similar to other studies carried out in Turkey (Sevinc *et al.*, 2008), Portugal (Ribeiro *et al*., 2013) and Israel using IFAT (Shkapt *et al*., 1998). However previous studies conducted in Spain showed higher values (Habela *et al*., 2000; Olmeda *et al*., 2001; García-Bocanegra *et al*., 2012).

Seroprevalence for *B. caballi* was 2.8% which is in accordance with the results around 2%, obtained in Greece (Kouam *et al*., 2010) and in Turkey (Sevinc *et al*., 2008) but is lower than than other results obtained in Spain (García-Bocanegra *et al.*, 2012) and Portugal (Ribeiro *et al*., 2013).

A possible explanation to these great differences might be related to the study population and assays characteristics, indeed, personal observation confirmed that IFAT seems to detect more positive than ELISA when analysing the same population.

Gender was a confirmed risk factor. Similar to the result obtained by other authors, geldings and females showed more positivity to EP than males: this could be due to management characteristics, since geldings and mares usually spend more time in pastures while stallions are kept in more controlled places (Sevinc *et al.*, 2008; Moretti *et al*., 2010;

Sumbria *et al*., 2017).

Seroprevalence positivity to *T. equi* seems to increase with age; this could be due to the persistence of antibodies (de Waal, 1992). These observations are in accordance with other authors (Rüegg *et al*., 2007; Kouam *et al*., 2010; Veronesi *et al*., 2010; García-Bocanegra *et al*., 2012; Zanet *et al*., 2017).

Significant differences due to breed were found for *T. equi*. Mixed breed horses and native breed horses showed higher values than foreign breed horses. These differences could be due to different management conditions, even if other authors have mentioned differences in susceptibility of different breeds (Sevinc *et al*., 2008, Moretti *et al*., 2010; Abutarbush *et al*., 2012; Steinman *et al*., 2012). All horses tested in this study were healthy and asymptomatic although they were seropositive, suggesting a tolerance to parasites and resistance to the disease. Some studies have been conducted about the diversity of genes for immune functions in the major histocompatibility complex and the ability to adapt in equids (Vranova *et al.*, 2011). Mixed breeds are usually very robust, native breeds are more adapted to the local environment, weather conditions and lacking feedstuffs and they are more resistant to the endemic pathogens they coexist with.

Significant differences related to the external antiparasite treatment were found. Oppositely to what expected, seroprevalences were higher in treated animals. A possible explanation could be a wrong administration of the product, in dose size and/or interval.

Regarding coat's colour, higher prevalences in lighter coloured animals were found significant for both parasites. Observations about the influence of coat's colour have been described by Aharonson-Raz *et al.* (2014).

Horse activity significantly influenced the prevalences. According to what other authors observed, sport horses showed the lowed prevalence (Kouam *et al.*, 2010; Abutarbush *et al*., 2012; Zanet *et al*., 2017).

Access to pasture and ticks presence on the animals were significant variables in the univariate test for *T. equi* seroprevalence. Horses kept outdoor were more positive to *T. equi* ELISA than the others because of the higher exposure to ticks. Similar observations were reported by other authors (Kouam *et al*., 2010, García-Bocanegra *et al*., 2012, Moretti *et al*.,

2010, Abutarbush *et al*., 2011; Steinman *et al*., 2012; Sumbria *et al.*, 2017).

Cohabitation with other species was a significant factor for *T.equi,* with higher prevalences observed in equids in contact with ruminants, dogs and wild species as already described by Criado-Fornelio *et al*. (2003), Camacho *et al*. (2005), Buttler *et al*. (2012), Abutarbush *et al*. (2012) and Sumbria *et al*. (2017)and due to the low host specificity of ticks.

Altitude also was a significant factor in the univariate model for *T. equi*, showing the seroprevalence tests more positives in the group of horses living at 250-600 m. These data could suggest that, as Shchuchinova *et al*. (2015) observed for tick-borne encephalitis, the intermediate range of altitude presents the highest diversity of Ixodid species.

Land cover showed to be a significant factor in the univariate analyses for *T. equi* indicating a higher positivity in animals living in mixed zones (with no dominance of a particular land cover category) than in crops areas. These results are in agreement with those reported by Vanwambeke *et al*. (2010), showing that arable fields, or patches of forests surrounded by agricultural lands, have a negative impact on vector-borne diseases. In the univariate model the planosol group showed the highest number of positives. Our data indicate that the differences in seroprevalence are influenced by abiotic characteristics and their interactions. These variables (altitude, land cover and soil) are also related to ticks distribution, abundance and behaviour. Climate, microclimate, humidity, temperature of soil and its pore size, altitude effects on climatic conditions, urbanization and adaptation of ticks to new environments and finally presence of the host have been described by Pfäffle *et al*. (2013), Steinman *et al*. (2012) and Sumbria *et al*. (2017). All these factors are related to the ticks presence, which is required for the stable maintenance of the parasites (Scoles & Ueti, 2015).

Table 1. Results of univariate analyses for *Theileria equi* ELISA and *Babesia caballi* ELISA. (N= number of samples tested; CI= confidence interval; p= p value). p value < 0.05 was considered significant.

			***T. equi* ELISA**			***B. caballi* ELISA**		
Variables	**Category**	**N**	**Positives (%)**	**CI 95%**	**p**	**Positives (%)**	**CI 95%**	**p**
Species	donkeys	40	12.25	(5-26)	0.089	5	(1-17)	0.311
	horses	139	25.2	(19-33)		2.2	(1-6)	
Horse breed	Foreign	31	12.9	(5-29)	0.041	0	(0-11)	0.571
	Native	101	26.7	(19-36)		3.3	(1-8)	
	Mixed	7	57.1	(25-84)		4	(1-20)	
Sex	Geldings	44	43.2	(30-58)	0.001	6.8	(2-18)	0.174
	Female	77	16.9	(10-27)		1.3	(0-7)	
	Male	58	13.8	(7-25)		1.7	(0-9)	
Age (years)	≤6	79	7.6	(4-16)	>0.0001	3.8	(1-11)	0.490
	7-12	62	30.6	(21-43)		3.2	(1-11)	
	>12	38	39.5	(26-55)		0	(0-9)	
Colour coat	light	64	34.4	(24-47)	0.004	6.3	(2-15)	0.036
	dark	115	15.7	(10-23)		0.9	(0-5)	
Activity	Recreation	41	14.6	(7-28)	<0.0001	4.9	(1-16)	0.383
	Sport	66	12.1	(6-22)		0	(0-6)	
	Police	47	46.8	(33-61)		4.3	(1-14)	
	Breeding	25	16	(6-35)		4	(1-20)	
Antiparasitic treatment	No	80	13.8	(8-23)	<0.0001	1.3	(0-7)	0.526
	Yes	52	13.5	(7-25)		3.8	(1-13)	
	Random	47	46.8	(33-61)		4.3	(1-14)	
Pasture	No	96	12.8	(7-21)	0.001	2.4	(1-8)	1.000
	Yes	85	32.9	(24-43)		3.2	(1-9)	
Tick presence	No	25	4	(1-20)	0.018	2.6	(1-6)	0.533
	Yes	154	25.3	(19-33)		4	(1-20)	
New introduction	No	171	21.6	(16-28)	0.293	2.9	(1-7)	1.000
	Yes	8	37.5	(14-69)		0	(0-32)	
Other species	Yes	132	13.6	(9-21)	<0.0001	2.3	(4.3)	0.607
	No	47	46.8	(33-61)		1-6	(1-14)	
Altitude (m)	150-600	47	46.8	(33-61)	<0.0001	4.3	(1-14)	0.607
	>600	132	13.6	(9-21)		2.3	(1-6)	
Land cover	50-75% crops	49	8.2	(3-19)	0.005	3.1	(1-9)	1.000
	Mixed	130	27.7	(21-36)		2.4	(1-8)	
Soil	Eutric Cambisol	40	12.5	(5-26)	<0.0001	5	(1-17)	0.530
	Dystrict cambisol	44	2.3	(0-12)		2.3	(0-12)	
	Xerosol	43	20.9	(11-35)		0	(0-8)	
	Planosol	52	48.1	(35-61)		3.8	(1-13)	

Conclusions

The present study shows the seroprevalences for both parasites. Several risk factors associated to the host and the environment were significantly related to the positivity for EP, confirming previous studies in the Mediterranean area, although further investigations about the influence of environmental factors on the tick ecology in Europe are needed.

These results confirm Central Spain as an enzootic area and warn about the need to implement adequate management practices such as tick control programmes and improve efficacy of laboratory diagnosis to identify carriers and to prevent the spread of the disease.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-profit sectors. Thanks to the animal holders, to the National Police Veterinary Department staff for their availability and help, to the Asociación Amigos del Burro and to doctors Mª Cruz Llorente, Isabel Martínez and José Luis García.

Conflict of interest

The author declares no conflict of interests.

Author contribution

Blood collection, ELISA tests performance, selection of risk factors, data analysis and manuscript redaction.

References

Abutarbush, S.M., Alqawasmeh, D.M., Mukbel, R.M., Al-Majali, A.M. Equine Babesiosis: Seroprevalence, Risk Factors and Comparison of Different Diagnostic Methods in Jordan. *Transbound. Emerg. Dis.* **2012**.59, 72–78.

Aharonson-Raz, K., Rapoport, A., Hawari, I.M., Lensky, I.M., Berlin, D., Zivotofsky, D., Klement, E., Steinman, A. Novel description of force of infection and risk factors associated with *Theileria equi* in horses in Israel and in The Palestinian Authority. *Ticks Tick. Borne. Dis.***2014** 5, 366–372.

Balkaya, I., Utuk, A.E., Piskin, F.C. Prevalence of *Theileria equi* and *Babesia caballi* in donkeys from

eastern Turkey in winter season. *Pak. Vet. J.,* **2010,** 30 (4): 245-246.

Butler, C.M., Sloet van Oldruitenborgh-Oosterbaan, M.M., Stout, T.A.E., van der Kolk, J.H., van den Wollenberg, L., Nielen, M., Jongejan, F., Werners, A.H., Houwers, D.J. Prevalence of the causative agents of equine piroplasmosis in the South West of The Netherlands and the identification of two autochthonous clinical *Theileria equi* infections. *Vet. J*. **2012.** 193, 381–385.

Camacho, A.T., Guitian, F.J., Pallas, E., Gestal, J.J., Olmeda, A.S., Habela, M.A., Telford, S.R., Spielman, A. *Theileria (Babesia) equi* and *Babesia caballi* infections in horses in Galicia, Spain. *Trop. Anim. Health Prod*. **2005.** 37, 293–302.

Chahan, B., Zhang, S., Seo, J.-Y., Nakamura, C., Zhang, G., Bannai, H., Jian, Z., Inokuma, H., Tuchiya, K., Sato, Y., Kabeya, H., Maruyama, S., Mikami, T., Xuan, X. Seroepidemiological evidence for the possible presence of *Babesia (Theileria) equi* and *Babesia caballi* infections in donkeys in western Xinjiang, China. *J. Vet. Med. Sci.* **2006**. 68, 753–5.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Saraña, A., Barba-Carretero, J.C. Molecular studies on *Babesia, Theileria* and *Hepatozoon* in southern Europe: Part I. Epizootiological aspects. *Vet. Parasitol.* **2003**. 113, 189–201.

De Waal, D.T. Equine piroplasmosis: a review. *Br. Vet. J*. **1992.** 148, 6–14.

García-Bocanegra, I., Arenas-Montes, A., Hernández, E., Adaszek, Ł., Carbonero, A., Almería, S., Jaén-Téllez, J.A., Gutiérrez-Palomino, P., Arenas, A. Seroprevalence and risk factors associated with *Babesia caballi* and *Theileria equi* infection in equids. *Vet. J*. **2013**. 195, 172–178.

Gizachew, A., Schuster, R.K., Joseph, S., Wernery, R., Georgy, N.A., Elizabeth, S.K., Asfaw, Y., Regassa, F., Wernery, U. Piroplasmosis in Donkeys A Hematological and Serological Study in Central Ethiopia. *J. Equine Vet. Sci.* **2013**. 33, 18–21.

Habela, A, Sevilla, G., Corchero, E., Pe, J., Extremadura, S. Diagnóstico ytratamiento de la Piroplasmosis equina. *Mundo Vet.* **2000**, noviembre, 62–68.

Kouam, M.K., Kantzoura, V., Masuoka, P.M., Gajadhar, A. a., Theodoropoulos, G. Genetic diversity of equine piroplasms in Greece with a note on speciation within *Theileria* genotypes (*T.*

equi and *T. equi-like*). *Infect. Genet. Evol.* **2010**. 10, 963–968.

Machado, R.Z., Toledo, C.Z.P., Teixeira, M.C. a, André, M.R., Freschi, C.R., Sampaio, P.H. Molecular and serological detection of *Theileria equi* and *Babesia caballi* in donkeys (Equus asinus) in Brazil. *Vet. Parasitol.* **2012**. 186, 461–5.

Mekibib, B., Manegerew, M., Tadesse, a., Abuna, F., Megersa, B., Regassa, a., Mekuria, S., Abebe, R. Prevalence of Haemoparasites and Associated Risk Factors in Working Donkeys in Abigudem and Kwiha Districs of Tigray Region, Northern Ethiopia. *J. Anim. Vet. Adv.* **2010**. 9 (17) 2249-2255.

Moretti, A., Mangili, V., Salvatori, R., Maresca, C., Scoccia, E., Torina, A., Moretta, I., Gabrielli, S., Tampieri, M.P., Pietrobelli, M. Prevalence and diagnosis of *Babesia* and *Theileria* infections in horses in Italy: A preliminary study. *Vet. J*. **2010**. 184, 346–350.

Oduori, D.O., Onyango, S.C., Kimari, J.N., MacLeod, E.T. A field survey for the seroprevalence of *Theileria equi* and *Babesia caballi* in donkeys from Nuu Division, Kenya. *Ticks Tick. Borne. Dis*. **2015**. 6, 683–688.

OIE - Terrestrial Animal Health Code Twentieth edition, **2011**.

Olmeda, **A.S**., Bösse R., Ndjeng P., Sanmartín H., Meana A., Babesiosis equina: serodetección por inmunofluorescencia en animales sintomáticos y clínicamente sanos. V Simposium ibérico sobre ixodoidea y enfermedades transmitidas. Madrid 27-29 septiembre de **2000**: 79-80

Olmeda A.S., Ndenjg P., Mateos A., Meana A., Dinámica estacional comparativa de la seroprevalencia de la babesiosis equina entre la zona centro y oriental de la Península Ibérica. VII Congreso ibérico de Parasitología, 18-21 septiembre **2001** en Oporto (Portugal): 165

Pfäffle, M., Littwin, N., Muders, S. V., Petney, T.N. The ecology of tick-borne diseases. *Int. J. Parasitol.* **2013**. 43, 1059–1077.

Ribeiro, A.J., Cardoso, L., Maia, J.M., Coutinho, T., Cotovio, M. Prevalence of *Theileria equi, Babesia caballi,* and *Anaplasma phagocytophilum* in horses from the north of Portugal. *Parasitol. Res.* **2013**. 112, 2611–2617.

Rüegg, S.R., Torgerson, P., Deplazes, P., Mathis, A. Age-dependent dynamics of *Theileria equi* and *Babesia caballi* infections in southwest Mongolia based on IFAT and/or PCR prevalence data from domestic horses and ticks. *Parasitology.* **2007**. 134, 939–47.

Scoles G., **Ueti M.W**. Vector ecology of equine piroplasmosis. *Annu. Rev. Entomol.* **2015,** 60, 561-580.

Shchuchinova LD, Kozlova IV, Zlobin VI: Influence of altitude on tick-borne encephalitis infection risk in the natural foci of the Altai Republic, Southern Siberia. *Ticks Tick Borne Dis.* **2015**, 6:322–329.

Shkap, V., Cohen, I., Leibovitz, B., Savitsky, Pipano, E., Avni, G., Shofer, S., Giger, U., Kappmeyer, L., Knowles, D. Seroprevalence of *Babesia equi* among horses in Israel using competitive inhibition ELISA and IFA assays. *Vet. Parasitol.* **1998.** 76, 251–259.

Sevinc, F., Maden, M., Kumas, C., Sevinc, M., Ekici, O.D. A comparative study on the prevalence of *Theileria equi* and *Babesia caballi* infections in horse sub-populations in Turkey. *Vet. Parasitol.* **2008**. 156, 173–177.

Steinman, A., Zimmerman, T., Klement, E., Lensky, I.M., Berlin, D., Gottlieb, Y., Baneth, G. Demographic and environmental risk factors for infection by *Theileria equi* in 590 horses in Israel. *Vet. Parasitol*. **2012.** 187, 558–562.

Sumbria, D., Singla, L.D., Sharma, A., Bal, M.S., Randhawa, C.S. Molecular survey in relation to risk factors and haemato-biochemical alteration in *Theileria equi* infection of equines in Punjab Province, *India. Vet. Parasitol. Reg. Stud. Reports.* **2017.** 8, 43–50.

Tefera, M., Worku, A., Tolosa, T., Bitew, M. Prevalence and risk factors for donkey babesiosis in and around Debre Zeit, central Ethiopia. *Vet. Res.* **2011.** 4 (2): 56-60.

Thrusfield, M. Veterinary Epidemiology, 3rd Edition. **2007.** Wiley-Blackwell, USA, 624 pp.

Vanwambeke SO, Sumilo D, Bormane A, Lambin EF, Randolph SE: Landscape predictors of tick-borne encephalitis in Latvia: land cover, land use, and land ownership. *Vector Borne Zoonotic Dis.* **2010.**10:497–506.

Veronesi, F., Laus, F., Moretta, I., Fioretti, D.P., Spaterna, A., Tesei, B., Moretti, A. Prevalenza di *Babesia caballi* e *Theileria equi* in cavalli di razza tolfetana. *Ippologia*. **2010.** 21, 3–9.

Vranova, M., Alloggio, I., Qablan, M., Vyskocil, M., Baumeisterova, A., Sloboda, M., Putnova, L., Vrtkova, I., Modry, D., Horin, P. Genetic diversity of the class II major histocompatibility DRA locus in European, Asiatic and African domestic donkeys. *Infect. Genet. Evol*. **2011**. 11, 1136–1141.

Zanet S., Bassano, M., Trisciuoglio, A., Taricco, I., Ferroglio, E. Horses infected by Piroplasms different from *Babesia caballi* and *Theileria equi* : species identification and risk factors analysis in Italy. *Vet. Parasitol.* **2017.**

STUDY 2.2

Molecular and serological detection of *Theileria equi* and *Babesia caballi* infections in Equids in areas of Central-Southern Italy.

Abstract

Serum samples were collected from 177 donkeys and 673 horses in Central-Southern Italy and examined by competitive-inhibition ELISA (cELISA) to evaluate the seroprevalence for *B. caballi* and *T. equi*. Blood samples from donkeys (169) and seropositive horses (263) were tested also by PCR to evaluate the potential carrier status of the animals that harbour a specific infectious agent without discernible clinical disease and serves as a potential source of infection. The following parameters were chosen to evaluate the risk factors related to equine piroplasmosis in horses: gender, age, breed, external parasite treatment, access to pasture, altitude of the farm, land cover, province location, climatic zone and soil type. In donkeys, due to the lack of data only risk factors related to origin, gender, breed and age were evaluated. Univariate and multiple logistic-regression models were conducted.

In horses, the overall seroprevalence for *T. equi* was 38.9% (268/673), and for *B. caballi,* 8.9% (60/673); 70.3% of the tested samples in PCR (185/263) were positive to *T. equi* and 10.3% (27/263) to *B. caballi.* The multiple logistic-regression model indicated that the risk factors associated with *T. equi* seroprevalence were increasing age, gender, breed, access to pasture, land cover province location and soil type. For *B. caballi* seroprevalence, the risk factors were the same except for climatic zone instead of age. Risks factors to *T. equi* PCR positivity were decreasing age and soil, and for *B. caballi* PCR soil and climatic zone. In donkeys, *T.equi* seroprevalence detected by ELISA was 54.8% (97/177); no seropositive samples for *B. caballi* were observed. PCR tests showed a 59.8% (101/169) and 5.9% (10/169) positivity for *T. equi* and *B.caballi,* respectively. Risk factors associated with *T. equi* seropositivity and PCR were gender and province, respectively.

Twenty-six adult free-living ticks were collected by drag-flag in an area were many seropositive and carriers had been detected. Fifteen of those, identified as *Dermacentor marginatus,* were positive to a *T. equi* PCR test. No tick was positive to *B. caballi* PCR test.

These results confirm the status of Italy as an endemic country and indicate the need

to improve control programmes to identify carriers and to prevent the spread of the disease.

Introduction

Equine piroplasmosis (EP) is a tick-borne disease caused by *Babesia caballi* and *Theileria equi,* which affects equids (horses, mules, donkeys and zebras). Both parasites are transmitted by ticks of the genera *Dermacentor, Rhipicephalus* and *Hyalomma.* The disease is endemic in many tropical and sub-tropical countries and causes sub-clinical infections, but also clinical forms and death. In donkeys, chronic forms are more frequent. All characteristics of the disease in the donkey has been described by Kumar *et al.* (2009). Diagnostics can be performed by stained blood identification, serological tests such as complement fixation test, indirect fluorescent antibody test (IFAT), ELISA or PCR methods. *Piroplasmosis* is a major constraint to the *international* movement of equines, whichseriously affects the horse industry (OIE 2011). Donkey husbandry is becoming more and more prosperous, with a strong growth of the sector. In many countries, donkeys are an essential tool in crop areas or used as beasts of burden due to their strength, resistance and size. On the other hand, in Europe, the agricultural sector has suffered a strong decrease in the last decades, which almost lead to the progressive extinction of the species. Fortunately, in the last years, other characteristics of these animals have been recognised and now they are increasingly used in fields such as assistant animal therapy (onotherapy) or milk production (e.g. donkey milk is suitable for children with allergic reactions to cow milk).

In horses, previous studies conducted in Italy showed different EP seroprevalences detected using IFAT ranging from 0.3% (Grandi *et al.*, 2011) to 56% (Moretti *et al.*, 2010) for *B. caballi*, and from 8.2% (Grandi *et al.*, 2011) to 50.48% (Moretti *et al.*, 2010) for *T. equi*. When using PCR, a wide range of results were observed: from 0% (Grandi *et al.*, 2011) to 6% (Laus *et al.*, 2013) for *B. caballi,* and from 11.7% (Laus *et al.*, 2013) to 33% (Grandi *et al.*, 2011) for *T. equi*.

In Italy, donkey piroplasmosis studies have been developed by Veronesi *et al.* (2014), Piantedosi *et al.* (2014) and Laus *et al.* (2015), plus additional studies restricted to few animals (Nardoni *et al*., 2007) or to a geographical area (Giudice *et al.*, 2007).

The objective of this study was to evaluate the serological and parasitic prevalences and risk factors in asymptomatic horses and in healthy donkeys from two milking farms in Central-Southern regions of Italy and to investigate the risk factors associated.

Materials and methods

Field study area and sampling

This study involved the equine population in central Italy. There are no reliable data about the horse size population in the area, so the calculation of the sample size was based on an expected prevalence of 50% in an infinite population, a confidence interval of 95%, and an absolute accuracy of 5%. The minimum number of samples required resulted in 384, so 673 horse samples from seven Italian provinces belonging to Latium and Campania Regions were included in the study.

Whole blood and EDTA samples were collected. Sera were obtained by centrifugation for 10 minutes at 358 g; then stored at –20°C and –80°C until used.

To evaluate the risk factors related to EP data, the following parameters were registered: region and province; sex (male / female); age (young ≤6; adult 7-12; senior >12 years old); breed was categorized in three groups (foreign breeds, Italian breeds, and mixed breed and mules); external parasite treatment (yes / no); access to pasture (yes / no) and province location (coastal/inland). Using a GPS system, the geographic location of the farms was determined, which resulted in additional variables being added to the risk factors evaluation file, such as altitude (≤ 150 meters; 151-600 meters; > 600meters); land cover (> 75% forest; crops 50-75%; 50-75% forest; mixed, with no dominant land cover); climate zone based on length of growing period (LGP), which is the period (in days) during a year when precipitation exceeds half the potential evapotranspiration (humid LGP 270-365 days, sub-humid LGP 180-269 days; moist-semiarid LGP 120-179 days); soil type (eutric cambisol with a base saturation of 50 % or more; dystrict cambisol with a base saturation of less than 50 %; andosol which are composed of vitric, volcanic ash, cinders and xerosol a kind of sub-arid soil).

Land cover, climate and soil information were obtained using the geographic coordinates in the interactive maps available at FAO website http://www.fao.org/geonetwork/srv/en/main.home.

One hundred and seventy seven samples from asymptomatic donkeys from two milking farms located in Rome (Latium) and Salerno (Campania) provinces (129 and 48 animals, respectively) were analysed. The Latium asymptomatic group is located in a 57 hectares area in the North of the region, and it is composed by native Italian breeds (Amiatino, Martina Franca donkey, Viterbese, and Ragusano). Although a regular vaccination protocol is not established on the farm, deworming products are administered periodically and molecules rotated. Official controls against infectious anaemia, equine arteritis, contagious equine metritis, dourine and brucellosis are performed. Dams are milked 3 times a day. No production decrease or changes in animal condition were observed. Anamnestic data about age, sex, breed and coat colours from this group were collected.

To investigate the presence of piroplasms in the vectors, 26 adult free living ticks were collected by drag-flag method in the Monti Aurunci Nature Park, placed between two Latium regions, range coordinates 41°21'N 13°40'E, where many serological and PCR positives have been detected. After identification, ticks were conserved in 70% ethanol solution at -80°C.

Serological tests

Two commercial competitive enzyme-linked immunosorbent assays (cELISA) were employed: *Babesia equi* Antibody test kit VMRD® and *Babesia caballi* Antibody test kit VMRD®; following the manufacturers' instructions.

Tick identification

Free living ticks were identified according to taxonomic keys described by Walker *et al.* (2003).

Biomolecular tests

EDTA blood of seropositive animals (263 samples) was examined by two real time PCRs protocols described by Kim *et al.* (2010) for *T. equi* and Bhoora *et al*. (2010), for *B. caballi.*

DNA extraction.

DNA blood extraction was performed using the automated robotic workstation QIAcube HT (Qiagen, GmbH, Hilden, Germany) and the QIAamp cador Pathogen Mini kit (Qiagen) according to the manufacturers' instructions. The DNA was eluted in 150 µl of buffer AVE -RNase-free water with 0.04% NaN_3 (Sodium azide) and stored at -80°C.

DNA extraction from adult ticks was performed using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) following a modified protocol for tissues with the following alterations: after 3 hours incubation in 180 µl buffer ATL at 56°C with 20 µl proteinase K, ticks were homogenized mechanically and incubated 2 hours in buffer AL at 70°C. The DNA was eluted in 100 µl of buffer AE. DNA yield was determined with a spectrophotometer (Eppendorf BioPhotometer, Eppendorf AG, Hamburg).

Real time for Theileria equi *and* Babesia caballi *from literature*

Real time PCR 18S rRNA *B. caballi* amplified a 95bp fragment in the V4 hypervariable region of 18S rRNA gene of *B. caballi*. Primers and probe employed (F: Bc-18SF402; R: Bc-18SR496; Probe: TaqMan MGB™ probe (FAM-MGB), Bc-18SP) were those reported in literature (Bhoora e*t al*., 2010). The real time 18S rRNA *T. equi* amplified an 81bp fragment in the V4 hypervariable region of 18S rRNA gene. Primers and probe employed (F: Be18SF; R: Be18SR) TaqMan probe (VIC-TAMRA, Be 18SP) were those reported in literature (Kim *et al*., 2008). For both real time PCRs, TaqMan® Universal PCR Master Mix kit (A.Biosystems, Foster City, CA, USA) was used. The positive controls were constituted by plasmid vectors pCRII®-TOPO TA Cloning® Invitrogen, Carlsbad, CA, USA) in which the targets of the real time PCR *T. equi* and *B. caballi* have been cloned.

All real time PCRs were carried out using ABIPRISM 7900 HT Sequence Detection System (A. Biosystems).

Tick PCR protocols

During preliminary tests, all the primers described before, hybridised tick genome, so other primers were selected to avoid this problem. In detail two protocols for *B. caballi* called BC48 nested PCR and RAP end point PCR and one protocol for *T. equi* called EMA end Point PCR were conducted. Methods are described below.

-BC48 nested PCR

The target for BC48 nested PCR was the BC48 gene coding for a 48KDa merozoite protein appointed BC48 and belonging to the complex rhoptry protein. The primers used for the first PCR were BC48F1/BC48R3 (Ikadai *et al.*, 1999; Battsetseg *et al.*, 2002) and amplified a 530 bp fragment of BC48 gene, while the primers for the second PCR were BC48F11/BC48R31 and amplified an internal band of 430 bp (Battsetseg *et al.*, 2001; Battsetseg *et al.*, 2002).

-RAP end point PCR

The target for RAP end point PCR was a fragment of 825 bp internal at the rhoptry-associated protein (RAP1) gene and the primers used were BC-RAP2F/ BC-RAP2R (Bhoora *et al*., 2010).

-EMA end point PCR

The target for EMA end point PCR was a 268 bp fragment internal at merozoite antigen 1 (EMA-1) gene coding for a major parasite surface antigen and the primers used were EMA-5/EMA-6 (Battsetseg *et al.*, 2002).

In all protocols AmpliTaq Gold® DNA Polymerase (A. Biosystems, Life Technologies, Austin, Tx, USA) to prepare the master mix and the GeneAmp® PCR System 9700 (A. Biosystems, Foster City, CA, USA) were used. PCR products were visualized after electrophoresis in a 1.5% Tris-Boric Acid-EDTA agarose gel and stained with GelRed 10,000X (Biotium, Hayward, CA, USA).

Sequencing

On horse samples showing a low real time PCR Ct (<24), a nested PCR protocol amplifying the hypervariable V4 region of the 18rRNA gene was performed to estimate the specificity of the results. This protocol has products of approximately 430bp and 390bp for *Theileria* and *Babesia* species, respectively (Nagore *et al.,* 2004). The master mix for PCR1 and PCR2 were constituted using AmpliTaq Gold® DNA Polymerase (A. Biosystems, Life Technologies, Austin, Tx, USA) and was performed using the GeneAmp® PCR System 9700 (A. Biosystems, Foster City, CA, USA). The thermal profile was optimised, in order to avoid non-specific reactions, changing the annealing temperature from 51°C, as indicated in the literature, to 54°C.

PCR products were visualized after electrophoresis in a 1.5% Tris-Boric Acid-EDTA agarose gel and stained with GelRed 10,000X (Biotium, Hayward, CA, USA).

For ticks samples, to confirm the positive results, the product of the above-described PCRs, were sequenced.

All the products were recovered from agarose gel using the QIAquick® PCR Purification kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions and

sequenced using the PCR primers of second PCR with the BigDye Terminator Cycle Sequencing Ready Reaction kit, version 3.1 (PerkinElmer, A. Biosystems, Foster City, CA, USA) in an automated sequencer (3500 Genetic Analyzer, A. Biosystems, Foster City, CA, USA). The nucleotide sequences obtained were analysed using the Genetic Analyzer Sequencing v5.4 (A. Biosystems, Foster City, CA, USA). Sequence homology were performed using the Basic Local Alignment Search Tool (BLAST) by comparing them to sequences of equine piroplasms in NCBI GenBank (http://www.ncbi.nlm.nih.gov). Sequences showing homology and "query coverage" >98% were chosen.

Statistical analysis

Serological and parasitic prevalence and their 95% confidence intervals (95% CI) were calculated as shown in the literature in terms of the area of study, region and for each risk factor investigated. The relationships between explanatory variables and seropositivity for piroplasms were tested in two steps. First, all associations between serological and parasitic prevalence against *T. equi* and *B. caballi* and the possible risk factors were evaluated by Chi square or Fisher's exact test. Secondly, the factors that were significative at first analysis (p values <0.05) were included in a multiple logistic regression model using a non-automatic backward selection of variables. An initial model was obtained using all potential explanatory variables, then variables with a non-significant p value were sequentially deleted. The model was re-run until all remaining variables presented statistically significant values (likelihood-ratio Wald's test, $p < 0.05$) and a potential causal relationship with the response variable existed. A p value <0.05 was considered statistically significant. STATA SE12.0 software for Windows was used for all statistical analyses (StataCorpLP, Texas, USA).

Results

Horses results

T. equi *seroprevalence*

Serological prevalences and correspondent IC 95% for each variable are shown in Table 1. The overall seroprevalence for *T. equi* was 39.8% (268/673, CI 95%: 36-44%).

The following parameters resulted significant (p<0.05) in the univariate model: sex, age, breed, access to pasture, altitude, land cover, province location, and soil. There were differences between male and female prevalences, the latter being significantly higher

(p<0.001). Age was a significant variable: the older, the higher seroprevalences (p<0.01). Significant differences (p<0.01) were found regarding breed: the seroprevalence in the mixed breed and mules group was higher than in the pure breed groups; between the groups of pure breeds the native group showed a higher prevalence than the foreign one. Higher prevalence in horses with access to pasture was also significant (p<0.01). Altitude was a significant factor (p<0.01): the highest prevalence was found in the group at 150-600m (p<0.01). Prevalence was higher in the island provinces than in the coastal ones (p<0,001).

Differences among regions and provinces were significant in all the analyses, but since borders depend on political decisions rather than geographic characteristics, and furthermore these characteristics vary considerably within a province, these variables were not included in the multivariate analyses.

The final multivariate model included seven risk factors: age, land cover, access to pasture, breed, province location, sex and type of soil, (Table 3).

T. equi *biomolecular tests*

Presence of *T. equi* DNA was detected in 70.3% of samples (185/263; CI 64.6-75.5).

The following parameters resulted significant (P<0.05) in the univariate model: gender, age, breed, access to pasture, altitude, land cover, climatic zone, soil, and province.

The trends of prevalences related to the parameters were similar to those described for serology, with the exception of age and altitude. The percentage of positivity significantly decreased with age (<0.001), while increased with altitude (p<0.05). More details can be found in Table 2. The multivariate model included age, province location and soil as risk factors Table 4).

B. caballi *seroprevalence*

The overall seroprevalence was 8.9% (60/673; CI 95%: 7-11.3%). P

The following parameters were significant (P<0.05) in the univariate model: sex, breed, access to pasture, province location, altitude, land cover, climatic zone, and soil.

Females showed a significantly higher seroprevalence than males (0.001). Significant differences (p<0.001) due to the breed and access were similar to those described for *T. equi*. More details can be found in Table 1.

The multivariate analysis could not be executed for *B. caballi* seroprevalence due to the lack of positive animals for different variables.

B. caballi *biomolecular tests*

Babesia caballi PCR was positive for 10.3% of samples (27/263; CI 7.2-14.5).

The following parameters resulted significant (P<0.05) in the univariate model: age, climatic zone, province location and soil. Positivity decreases significantly with age (p<0.05). More details can be found in Table 2. Multivariate model for *B. caballi* PCR included climatic zone and soil as risk factors (Table 4).

Regarding coinfection, 56/673 animals were seropositive for both parasites while 23/263 animals were positive for *B. caballi* and *T. equi* PCR tests.

Donkeys results

The seroprevalence for *T. equi* was 54.8% (97/177), while for *B. caballi,* no positive samples were detected. PCR tests showed 5.9% (10/169) and 59.8% (101/169) prevalences for *Babesia* and *Theileria,* respectively. The PCR prevalence for *T. equi* seropositive donkeys was 76.3% (74/97). Prevalences and confidence intervals are described in Table 5, and by farm in Table 6. Significant differences due to this factor were found in the *T. equi* protocol PCR (65.3% Latium and 45.8% Campania).

The univariate analysis in the Latium group showed significant differences in sex groups for the *T. equi* ELISA prevalence (59.8% females and 35% males). No significant differences were found for *B.caballi* PCR. All prevalence values and factors included in the univariate analyses are described in Tables 7 and 8. Logistic regression was not possible due to univariate results.

Ticks results

All ticks were identified as *Dermacentor marginatus.* Fifteen of the 26 *D. marginatus* ticks resulted positive by EMA PCR. RAP and BC48 primers were used to detect *B. caballi* on ticks, but no positive results were confirmed by sequencing.

Table 1. Results of univariate analyses for *Theileria equi* ELISA and *Babesia caballi* ELISA. (N= number of samples tested; CI= confidence interval; p= p value). p value < 0.05 was considered significant.

			***T. equi* ELISA**			***B. caballi* ELISA**		
Variables	**Category**	**N**	**Positives (%)**	**CI 95%**	**p**	**Positives (%)**	**CI 95%**	**p**
Sex	Male	344	29.9	(25.3-35.0)	<0.001	2.9	(2.5-3.5)	<0.001
	Female	329	50.2	(44.8-55.5)		15.2	(11.7-19.5)	
Age (years)	≤6	214	31.8	(25.9-38.3)	<0.01	10.7	(7.3-15.6)	0.08
	7-12	237	43.9	(37.7-50.2)		11.0	(7.6-15.6)	
	>12	204	44.6	(37.9-51.5)		5.4	(3.0-9.4)	
Breed	Foreign	203	30.5	(24.6-37.2)	<0.01	2.5	(1.1-5.6)	<0.01
	Native	260	41.5	(35.7-47.6)		9.6	(6.6-13.8)	
	Mixed	210	46.7	(40.0-53.4)		14.3	(10.2-19.7)	
Antiparasitic treatment	No	475	37.9	(33.6-42.3)	0.073	7.6	(5.5-10.3)	0.06
	Yes	198	44.4	(37.7-51.4)		12.1	(8.3-17.4)	
Pasture	No	192	31.3	(25.1-38.1)	<0.01	1.6	(0.5-4.5)	<0.01
	Yes	481	43.2	(38.9-47.7)		11.9	(9.3-15.0)	
Altitude (m)	<150	328	34.1	(29.2-39.4)	<0.01	1.2	(0.5-3.1)	<0.001
	150-600	275	47.3	(41.5-53.2)		20.0	(15.7-25.1)	
	>600	70	37.1	(26.8-48.9)		1.4	(0.3-7.7)	
Land cover	>75% forest	65	44.6	(33.2-56.7)	<0.01	3.1	(0.8-10.5)	<0.001
	50-75% crops	184	40.2	(33.4-47.4)		8.7	(5.4-13.7)	
	50-75% forest	116	26.7	(19.5-35.4)		0.9	(0.2-4.7)	
	Mixed	308	43.5	(38.1-49.1)		13.3	(10.0-17.6)	
Climatic zone	Humid	393	37.2	(32.5-42.0)	0.114	3.3	(1.9-5.6)	<0.001
	Sub-Humid	260	42.3	(36.5-48.4)		17.7	(13.5-22.8)	
	Moist-semiarid	20	60.0	(38.7-78.1)		5.0	(0.9-23.6)	
Soil	Eutric Cambisol	134	44.0	(35.9-52.5)	<0.001	2.2	(0.8-6.4)	<0.001
	Dystrict cambisol	216	31.5	(25.7-38.0)		1.4	(0.5-4)	
	Andosol	177	25.4	(19.6-32.3)		0	(0-2.1)	
	Xerosol	146	65.8	(57.7-73.0)		37.0	(29.6-45.1)	
Inland province	No	332	31.3	(26.6-36.5)	<0.001	4.2	(2.5-7.0)	<0.001
	yes	341	48.1	(42.8-53.4)		13.5	(10.3-17.5)	

Table 2. Results of univariate analyses for *Theileria equi* PCR and *Babesia caballi* PCR. (N= number of samples tested; CI= confidence interval; p= p value). p value < 0.05 was considered significant.

			***T.equi* PCR**			***B.caballi* PCR**		
Variables	**Category**	**N**	**Positives (%)**	**CI 99%**	**p**	**Positives (%)**	**CI 99%**	**p**
Sex	Male	100	61.0	(51.2-70.0)	<0.01	8.0	(4.1-15.0)	0.343
	Female	163	76.1	(69.0-82.0)		11.7	(7.6-17.5)	
Age (years)	≤6	67	83.6	(72.9-90.6)	<0.001	14.9	(8.3-25.3)	<0.05
	7-12	104	74.0	(64.9-81.5)		13.5	(8.2-21.3)	
	>12	81	61.7	(50.8-71.6)		3.4	(1.2-9.7)	
Breed	Foreign	61	57.4	(44.9-69.0)	<0.01	11.5	(5.7-21.8)	0.902
	Native	104	69.2	(59.8-77.3)		10.6	(6.0-18.0)	
	Mixed	98	79.6	(70.6-86.4)		9.2	(4.9-16.5)	
Antiparasitic treatment	No	182	68.1	(61.0-74.5)	0.239	8.2	(5.1-13.2)	0.105
	Yes	81	75.3	(64.9-83.4)		14.8	(8.7-24.1)	
Pasture	No	57	57.9	(41.1-73.0)	<0.05	5.3	(1.8-14.4)	0.219
	Yes	206	73.8	(65.2-80.8)		11.7	(8.0-16.7)	
Altitude (m)	<150	107	62.6	(53.2-71.2)	<0.05	5.6	(2.6-11.7)	0.065
	150-600	129	72.9	(64.6-79.8)		14.7	(9.6-21.9)	
	>600	27	88.9	(71.9-96.1)		7.4	(2.1-23.4)	
Land cover	>75% forest	30	86.7	(70.3-94.7)	<0.05	10.0	(3.5-25.6)	0.599
	50-75% crops	74	78.4	(67.7-86.2)		10.8	(5.6-19.9)	
	50-75% forest	32	59.4	(42.3-74.5)		3.1	(0.6-15.7)	
	Mixed	127	64.6	(55.9-72.3)		11.8	(7.3-18.6)	
Climatic zone	Humid	141	60.3	(52.0-68.0)	<0.001	5.7	(2.9-10.8)	<0.05
	Sub-Humid	109	81.7	(73.4-87.8)		16.5	(10.7-24.6)	
	Moist-semiarid	13	84.6	(57.8-95.7)		7.7	(1.4-33.3)	
Soil	Eutric Cambisol	59	64.4	(51.7-75.4)	<0.001	13.6	(7.0-24.5)	<0.05
	Dystrict	70	77.1	(66.0-85.4)		4.3	(1.5-11.9)	
	Andosol	44	43.2	(29.7-57.8)		2.3	(0.4-11.8)	
	Xerosol	90	82.2	(73.1-88.8)		16.7	(10.4-25.7)	
Inland province	No	332	55.9	(46.2-65.1)	<0.001	4.9	(2.1-11.0)	<0.05
	yes	341	79.5	(72.6-85.0)		13.7	(9.2-19.8)	

Table 3: Multivariate logistic analysis of risk factors for *Theileria equi* ELISA. (CI= confidence interval; p= p value; a=baseline). p value < 0.05 was considered significant.

***Theileria equi* ELISA**				
Variables	**Category**	**p**	**Odds ratio**	**CI 95%**
Sex	Male	a		
	Female	0.001	1.86	(1.27-2.71)
Age (years)	≤6	a		
	7-12	0.002	2.09	(1.32-3.31)
	>12	0.000	2.59	(1.61-4.16)
Breed	Foreign	a		
	Native	0.000	3.57	(1.99-6.41)
	Mixed	0.002	2.51	(1.38-4.55)
Pasture	No	a		
	Yes	0.004	2.22	(1.29-3.83)
Land cover	>75% forest	a		
	50-75% crops	0.170	0.56	(0.25-1.27)
	50-75%	0.020	0.42	(0.20-0.87)
	Mixed	0.564	0.75	(0.29-1.95)
Soil	Eutric	a		
	Dystrict	0.001	0.29	(0.14-0.60)
	Andosol	0.084	0.49	(0.21-1.10)
	Xerosol	0.002	3.16	(1.54-6.47)
Inland	No	a		
	Yes	0.005	2.5	(1.32-4.73)

Table 4: Multivariate logistic analysis of risk factors for *Theileria equi* and *Babesia caballi* PCR. (CI= confidence interval; p= p value; a=baseline). p value < 0.05 was considered significant.

***Theileria equi* PCR**				
Variables	**Category**	**p**	**Odds Ratio**	**CI 95%**
Age (years)	≤6	a		
	7-12	0.110	0.51	(0.22-1.16)
	>12	0.004	0.29	(0.12-0.66)
Soil	Eutric Cambisol	a		
	Dystrict cambisol	0.04	3.58	(1.49-8.66)
	Andosol	0.354	1.70	(0.55-5.29)
	Xerosol	0.001	4.55	(1.87-11.08)
Inland	No	a		
	Yes	0.007	2.91	(1.33-6.35)
***Babesia caballi* PCR**				
Variables	**Category**	**p**	**Odds Ratio**	**CI 95%**
Climatic	Humid	a		
	Sub-Humid	0.009	3.55	(1.38-9.16)
	Moist-semiarid	0.185	5.83	(0.42-79.17)
Soil	1	a		
	2	0.020	0.14	(0.03-0.74)
	3	0.091	0.16	(0.02-1.34)
	4	0.749	0.85	(0.31-2.30)

Sequencing

Forty horse samples were sequenced and showed homology (>98%) with the following GenBank registered sequences, indicated by their accession number.

Babesia caballi: EU888904.1, Z15104.1, EU888900.1, AB734386.2, EU642514.1, AB734387.2, AB734392.2, JX049130.1

Theileria equi : KC465785.1, JX177673.1, EU888906.1, KF559357.1, HM229407.1, DQ287951.1, AB515314.1, KM046921.1, KJ787765.1, KJ573370.1, JX679181.1, AB733379.2, JQ657703.1, KJ573372.1, JX679180.1, JX049129.1, KM046922.1, KJ787772.1, HM229408.1, KJ573374.1, AB515312.1, AB515309.1, GU361791.1, GU361790.1

Twenty-two donkey samples were sequenced, and products showed homology and "query coverage" from 98-100% with registered *T. equi* accession numbers in BLAST data base

AB515308.1, AB515310.1, AB515311.1, AB515315.1, AB733379.2, EU642507.1, JX679181.1, KF559357.1, KJ573374.1, KM046921.1.

Varieties "like" for *B. caballi* and *T. equi* similar to those described by Nagore *et al*. (2004) were found.

All ticks positive to EMA PCR test were sequenced and *T. equi* positive results were confirmed showing 100% homology to GenBank registered sequences JQ782603.1, U97167.1, U97168.1, AB015213.1, AB015214.1, AB015215.1, AB015216.1, AB015217.1, AB015218.1, AB015220.1.

Table 5: Prevalences in donkeys by different methods and CI= confidence interval:

Test	**N**	**Positives (%)**	**CI 95%**
B.caballi ELISA	177	0	(0-18)
T. equi ELISA	177	54.8	(47-62)
B.caballi PCR	169	5.9	(3-11)
T.equi PCR	169	59.8	(52-67)
T.equi PCR (seropositives)	74	76.3	(67-84)

Table 6: Prevalences of the two donkeys milking farms.

		Latium donkeys			**Campania donkeys**		
Test	**p**	**N**	**Positives (%)**	**CI 95%**	**N**	**Positives (%)**	**CI 95%**
B.caballi ELISA	-	129	0	(0-5)	48	0	(0-3)
T. equi ELISA	0.661	129	55.8	(47-64)	48	52.1	(38-66)
B.caballi PCR	0.154	121	6.6	(3-13)	48	4.2	(1-14)
T.equi PCR	0.020	121	65.3	(56-73)	48	45.8	(33-60)

Table 7: Results of univariate analyses for *Theileria equi* serological and molecular tests. (N= number of samples tested; CI= confidence interval; p= p value). p value < 0.05 was considered significant.

			***T. equi* ELISA**			***T. equi* PCR**		
Variables	**Category**	**N**	**Positives (%)**	**CI 95%**	**p**	**Positives (%)**	**CI 95%**	**p**
Sex	Male	20	35.0	(18-57)	0.036	75.0	(53-89)	0.285
	Female	90	59.8	(50-69)		63.3	(53-73)	
Age (years)	≤6	71	50.7	(39-62)	1.29	67.1	(56-77)	0.760
	7-12	37	59.5	(43-74)		61.1	(45-75)	
	>12	4	100.0	(51-100)		75.0	(30-95)	
Breed	Amiata	71	60.6	(49-71)	0.407	64.2	(55-76)	0.516
	Martina	20	40.0	(22-61)		63.2	(41-81)	
	Ragusano	3	66.7	(21-94)		100.0	(44-100)	
	Viterbese	6	50.0	(19-81)		50.0	(19-81)	
Coat	Bay	18	50.0	(29-71)	0.272	66.7	(44-84)	0.888
	Grey	17	47.1	(26-69)		58.8	(36-78)	
	Black	9	33.3	(12-65)		57.1	(25-84)	
	Grey Black-	67	62.7	(51-73)		67.2	(55-77)	

Discussion

In this study, 673 horse samples were examined from asymptomatic horses to determine the seroprevalence for EP and the positivity of seropositive samples (263) to PCR tests to determine the carrier status of the horses and the risk factors associated both to serological and PCR positivity.

Prevalence for *T. equi* was 39.8%.This result is similar to previous studies carried out in Italy using IFAT (Laus *et al*., 2013; Moretti *et al.*, 2010), where prevalences were 41% and 50.48%, respectively, and with other results described in the Mediterranean area using cELISA, i.e. 33.7% in Israel (Shkap *et al.*, 1998) and 50.3% in Spain (García-Bocanegra *et al*., 2012). However, this result is higher than the *T. equi* seroprevalence observed in horses from Central Spain in the study 2.1 (25.2%).

PCR positivity resulted 70.3%, while similar studies conducted in Italy showed a lower percentage of positivity (Grandi *et al.*, 2011, Moretti *et al.*, 2010). This difference could be attributed to the different method employed (end point vs real time), target choice (type and length), and number of samples examined.

Table 8: Results of univariate analyses for *Babesia caballi* molecular test. (N= number of samples tested; CI= confidence interval; p= p value). p value < 0.05 was considered significant.

***B.caballi* PCR literature protocol**				
Variables	**Category**	**Positives (%)**	**CI 95%**	**p**
Sex	Male	15.0	(5-36)	0.200
	Female	4.4	(2-11)	
Age (years)	≤6	7.1	(3-16)	0.204
	7-12	2.8	(0-14)	
	>12	25.0	(5-70)	
Breed	Amiata	5.6	(2-14)	0.243
	Martina Franca	10.5	(3-31)	
	Ragusano	33.3	(6-79)	
	Viterbese	0.0	(0-39)	
Coat	Bay	11.1	(3-33)	0.613
	Grey	5.9	(1-27)	
	Black	14.3	(3-51)	
	Grey Black-	4.5	(2-12)	

Seroprevalence for *B. caballi* was 8.9%, which is in accordance with the results of previous Spanish study 8.4% (García-Bocanegra *et al*., 2012), but higher than other results, i.e. around 2% obtained in Greece (Kouam *et al*., 2010), in Turkey (Sevinc *et al*., 2008) and Spain (see previous study 2.1). This can probably be explained due to the presence of a cluster of infection on the Aurunci Mountains. Italian studies using IFAT described by Laus *et al*. (2013) and Moretti *et al.* (2010) showed higher prevalence values (26% and 56%, respectively). A possible explanation to this great difference might be related to the study population and assays characteristics. Indeed, personal observation confirmed that IFAT seems to detect more positive than ELISA when analysing the same population.

PCR positivity to *B. caballi* was 10.3% which is higher than in other studies developed in Italy (Grandi *et al.*, 2011, Moretti *et al.*, 2010). The considerations described for *T. equi* PCR could explain this evidence.

Gender was a confirmed risk factor, with females showing more positivity to EP than males. This could be due to management characteristics, e.g. mares spend more time in pastures and usually stallions are kept in more controlled places. In the multivariate model,

females showed an OR of 1.8 respect to males in *T. equi* cELISA. Similar results were observed by other authors (Moretti *et al*., 2010; Sumbria *et al*., 2017).

Differences related to age were significant in the univariate analysis for *B. caballi* PCR and in both multivariate models for *T. equi* (ELISA and PCR). For *B. caballi,* positivity decreases with age. This could be explained by the fact that *B. caballi* could be completely eliminated in four years and antibodies are not lifelong (de Waal, 1992). Seroprevalence positivity to *T. equi* seems to increase with age, i.e. the older group shows an OR of 2.42 respect to the baseline. This could be due to the persistence of antibodies (de Waal 1992). These observations are in accordance with other authors (Kouam *et al.*, 2010; García-Bocanegra *et al*., 2012; Zanet *et al*., 2017).

However, for *T. equi*, the PCR OR decreases with age, probably due to the ability of the subject to control the parasitemia in the absence of reinfection, concomitant health problems or stress factors, maintaining it very low, maybe under the sensitivity limit of our method.

Significant differences due to breed were found in the univariate analyses for *B. caballi* seroprevalence and *T. equi* PCR. They were significant in the multivariate analysis for *T. equi* ELISA variable. Native breed horses and mixed breed ones showed 3 times and 3.16 times respectively more risk to be seropositive to *T. equi* cELISA than foreign breed horses. These differences could be due to different management conditions, even if other authors have mentioned differences in susceptibility of different breeds (Steinman *et al.*, 2012; Sevinc *et al,* 2008). All horses tested in this study were healthy and asymptomatic although they were carriers, suggesting a tolerance to parasites and resistance to the disease. Some studies have been conducted looking into the diversity of genes for immune functions in the major histocompatibility complex and the ability to adapt in equids (Vranova *et al.*, 2011). Mixed breeds are usually very robust. Native breeds are more adapted to the local environment, weather conditions and lacking feedstuffs and they are more resistant to endemic pathogens with which they coexist continuously. Further research is needed to clarify this.

In all the univariate analyses, non-significant differences were found related to the external parasite treatment, although with border line p values. Opposite to what we expected, seroprevalences were higher in the treated groups. A possible explanation could be a wrong administration of the product, in dose size and/or interval.

Access to pasture was a significant variable in the univariate model for *B. caballi* serological test and *T. equi* PCR and in the multivariate analysis for *T. equi* seroprevalence test. Horses kept outdoor were 2.7 times more positive to *T. equi* ELISA because of the higher exposure to ticks. Similar observations were reported by other authors (Kouam *et al*., 2010; García-Bocanegra *et al*., 2012; Shkap *et al*., 1998; Moretti *et al.,* 2010; Steinman, *et al.* 2012).

Altitude was a significant factor in the univariate model for both seroprevalence tests and *B. caballi* PCR, showing more positives in the group of horses living at 150-600 m. and for *T. equi* PCR (more positives in the >600m group), but this variable was excluded in the multivariate analysis. These data could suggest that, as observed by Shchuchinova *et al*. (2015) for tick-borne encephalitis, in the intermediate range of altitude is present the highest diversity of Ixodid species. The difference for *T. equi* PCR could be due to the limited number of samples tested in the highest altitude range.

Land cover showed to be a significant factor in the univariate analyses for *B. caballi* ELISA and *T. equi* PCR indicating respectively a higher positivity in animals living in mixed zones (with no dominance of a particular land cover category) and in areas with more than 75% forest. In the multivariate model of *T. equi* ELISA land cover was a significant variable, with a higher odd to be positive in the in mixed and >75% forest categories compared to the other two. These results are in agreement with those reported by Vanwambeke *et al*. (2010), showing that arable fields, or patches of forests surrounded by agricultural lands, have a negative impact on vector-borne diseases, while a high percentage of forest landcover has a positive effect.

The climate zone in the univariate analyses was significant for *B. caballi* ELISA and PCR and for *T. equi* PCR; in the multivariate analysis was significant for *B. caballi* PCR. For both *B. caballi* tests sub-humid zones (LGP 180-269 days) showed higher prevalences, with an OR of 3.5 compared to humid zone for PCR. It could be hypothesized that this climate zone coexist several conditions which favour the presence and the interactions among hosts and vectors.

However moist semiarid zones (LGP 120-179) showed high prevalence for *T. equi* PCR in the univariate analysis, although this result should be confirmed by including a wider number of samples for this climatic zone.

Soil was a significant variable in all the univariate multivariate models; for both

parasites and techniques, the xerosol group showed the highest number of positives. In the multivariate models for *T. equi* subject belonging to this group presented an OR between 2 and 2.9 respect to the eutric group, even if in the PCR model also the dystric group showed an OR of 2.6. In the multivariate model for *B. caballi* PCR dystric group showed and OR less than 1 compared to the baseline and no differences were detected between xerosol and the eutric group.

The inland provinces showed an OR respectively of 2.5 for seroprevalence and 2.9 for PCR positivity for *T. equi* with respect to the coastal provinces that could be attributed to a more suitable habitat for the vectors.

Gender, age, breed, altitude, land cover and soil were also significant variables for *T. equi* seroprevalence in the previous study conducted in Central Spain.

Our data indicate that the differences in seroprevalence and in presence of piroplasms are influenced by abiotic characteristics and their interactions. These variables (altitude, land cover, climatic zone and soil) are also related to ticks distribution, abundance and behaviour. Climate, microclimate, humidity, temperature of soil and its pore size, altitude effects on climatic conditions, urbanization and adaptation of ticks to new environments and finally presence of the host have been described by Pfäffle *et al*. (2013). All these factors are related to the ticks presence, which is required for the stable maintenance of the parasites (Scoles & Ueti, 2015).

In asymptomatic donkeys seroprevalence for *B.caballi* by ELISA was 0, which has been reported by Oduori *et al*. (2015) but it doesn't agree with other authors as García-Bocanegra et al. (2012) who described prevalences over 17% in Spain; the previous study (2.1) conducted on equids form Central Spain also showed a higher *B.caballi* serprevalence in donkeys (5%). *B. caballi* seroprevalence detected by IFAT described in Italy by other authors, Piantedosi *et al*. (2014), Veronesi *et al.* (2014) and Laus *et al.* (2015) ranged from 35.5 to 47.8%. PCR prevalence (5.9%) data are in accordance with those described by Laus *et al*. (2015) in Italy. Seroprevalence for *T.equi* by ELISA (54.8%) is higher than data reported by Garcia-Bocanegra *et. al* (2012) or data presented in the previous study of this thesis conducted in donkeys from Central Spain (12.25%) but lower than Machado *et al.* (2012) in Brazil (>70%). *T.equi* seroprevalence is also higher than IFAT prevalences registered by the three Italian authors above which ranged from 39-44%. *T. equi* parasite prevalence was 59.8%, which is close to data

reported by Machado *et al.* (2012) in Brazil, but higher than Laus *et al.* (2015) and Veronesi *et al.* (2014) detections.

The donkey group (177) was constituted by two milking farms from two different regions, all prevalence data are higher in Latium region than in Campania but just differences by *T.equi* PCR protocol were statistically significant. Risk factors analysed in the Latium milking farm were gender, which resulted a significant factor in the *T.equi* ELISA test showing higher prevalences in females. In the PCR tests for both parasites males presented higher prevalences but was not a significant value. However for Piantedosi *et al.* (2014) sex was just significant in *B.caballi* seroprevalence detected by IFAT. Related to age, higher prevalences appear in aged animals by different tests but it was not a statistically significant factor. Oduori *et al.,* 2015 didn't find age and gender significant risk factors. In our study no differences related to breed or coat colour were observed in any analyses.

Prevalences for *T. equi* were higher in donkeys than horses, 54.8% versus 39.8% in serology (p=0.0004) and 76.3% *vs*.70.3% in PCR (p=0.2921). For *B. caballi* due to the lack of serological positives by ELISA no conclusions can be taken because of the PCR values in donkeys are calculated on the overall population (5.9%) and in horses on the seropositive animals (10.3%); based on personal observations performing another study on the same donkey population using Immunofluorescence Antibody Test (IFAT) prevalences for *B. caballi* in serology and PCR on seropositives were higher in donkeys (18% vs. 8.9% p=0,0002 and 17% *vs.* 10.3% p=0.2403).

Piroplasms vectors can be *Dermacentor, Rhipicephalus* or *Hyalomma* (de Waal, 1992; Scoles & Ueti, 2015). Its presence in the territory of study has been detected with the collection of 26 free-living *Dermacentor marginatus*. This sampling is not statistically representative of the whole study area, as was performed in order to investigate a geographic cluster of high seroprevalence and PCR positivity for piroplasms. Ten of them were positive to *T. equi* PCR. Although the presence of *B. caballi* infected ticks (that can transmit transovarially and trans-stadially) was suspected in the investigated areas, it was not confirmed. The lack of positivity to *B. caballi* could be real or due to the low parasitic charge in the tick examined and the limit of sensitiveness of the two protocols. This does not exclude that the maintenance of the cluster of *B. caballi* could be attributed to other genera of ticks, already described as present in the country (Scoles and Ueti, 2015).

Conclusions

The present study shows the high seroprevalences and number of carriers for both parasites in horses, with a cluster located in the Latium region. Several risk factors associated to the host and the environment were significantly related to the positivity for EP, confirming previous studies in the Mediterranean area, although further investigations about the influence of environmental factors on the ticks ecology in Europe are needed. Donkey population in our study also showed high piroplasms prevalences in asymptomatic animals, characteristic of an endemic area this fact and the low number of samples brought for diagnostic suspicion could indicate the great resistance of these animals and the predomination of chronic or subclinic forms. Latium Region seems to show higher prevalences for both parasites, may be due to breeding practices and presence of the vectors. The carrier role of donkeys for the maintenance and spread of the infections in equids is evident. These data, and the presence of free living infected *Dermacentor marginatus*, recognized competent vectors, in an area where many seropositive animals and carriers have been detected, confirm the status of Italy as an endemic country and warn about the need to implement adequate management practices such as tick control programmes and improve efficacy of laboratory diagnosis, using both serological and biomolecular tests, to identify carriers and to prevent the spread of the disease.

Acknowledgements

This study was developed with funds from the IZSLT 16/11 project (provided by the Italian Ministry of Health). Thanks to doctors Deliberato and Magliano.

Conflict of interest

The authors declare no conflict of interests.

Author contribution

Blood collection from some animals, tick collection, ELISA tests, DNA extraction, PCRs performance, DNA purification, study of risk factors and manuscript redaction.

References

Battsetseg, B., Xuan, X., Ikadai, H., Bautista, J.L.R., Byambaa, B., Boldbaatar, D., Battur, B., Battsetseg, G., Batsukh, Z., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K.. Detection

of *Babesia caballi* and *Babesia equi* in *Dermacentor nuttalli* adult ticks. Int. J. Parasitol. **2001**. 31, 384–386.

Battsetseg, B., Lucero, S., Xuan, X., Claveria, F.G., Inoue, N., Alhassan, A., Kanno, T., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K. Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. *Vet. Parasitol.* **2002.** 107, 351–357.

Bhoora, R., Quan, M., Franssen, L., Butler, C.M., Van der Kolk, J.H., Guthrie, A.J., Zweygarth, E., Jongejan, F., Collins, N.E., Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. *Vet. Parasitol.* **2010.** 168, 201–211.

Bhoora, R., Quan, M., Zweygarth, E., Guthrie, A.J., Prinsloo, S. a., Collins, N.E., Sequence heterogeneity in the gene encoding the rhoptry-associated protein-1 (RAP-1) of *Babesia caballi* isolates from South Africa. *Vet. Parasitol*. **2010**. 169, 279–288.

De Waal, D.T. Equine piroplasmosis: a review. *Br. Vet. J*. 1992.148, 6–14.

García-Bocanegra, I., Arenas-Montes, A., Hernández, E., Adaszek, Ł., Carbonero, A., Almería, S., Jaén-Téllez, J.A., Gutiérrez-Palomino, P., Arenas, A. Seroprevalence and risk factors associated with *Babesia caballi* and *Theileria equi* infection in equids. *Vet. J*. **2013**. 195, 172–178.

Giudice, E., Marino, A.M.F., Ferrantelli, V., Vicari, D., Scimeca, I., Nicosia, S., Torina, A. *Theileria equi* and *Babesia caballi* infections in donkeys in Sicily. *Parassitologia.* **2007***.* 49, suppl 1, 88.

Gizachew, A., Schuster, R.K., Joseph, S., Wernery, R., Georgy, N.A., Elizabeth, S.K., Asfaw, Y., Regassa, F., Wernery, U. Piroplasmosis in Donkeys A Hematological and Serological Study in Central Ethiopia. *J. Equine Vet. Sci*. **2013**. 33, 18–21.

Grandi, G., Molinari, G., Tittarelli, M., Sassera D., Kramer L., 2011 Prevalence of *Theileria equi* and *Babesia caballi* infection in horses from Northern Italy. *Vector Borne Zoonotic Dis*. **2011.** 11, 955-956.

Ikadai, H., Xuan, X., Igarashi, I., Tanaka, S., Kanemaru, T., Nagasawa, H., Fujisaki, K., Suzuki, N., Mikami, T. Cloning and expression of a 48-kilodalton *Babesia caballi* merozoite rhoptry

protein and potential use of the recombinant antigen in an enzyme- linked immunosorbent assay. *J. Clin. Microbiol.* **1999**. 37, 3475–3480.

Kim, C.M., Blanco, L.B.C., Alhassan, A., Iseki, H., Yokoyama, N., Xuan, X., Igarashi, I. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. *Vet. Parasitol.* **2008.** 151, 158–163.

Kouam, M.K., Kantzoura, V., Masuoka, P.M., Gajadhar, A. a., Theodoropoulos, G.,. Genetic diversity of equine piroplasms in Greece with a note on speciation within *Theileria* genotypes (*T. equi and T. equi-like*). *Infect. Genet. Evol*. **2010**. 10, 963–968.

Kumar, S., Kumar, R., Sugimoto, C. A perspective on *Theileria equi* infections in donkeys. *Jpn. J. Vet. Res*. **2009**. 56, 171–180.

Laus, F., Veronesi, F., Passamonti, F., Paggi, E., Cerquetella, M., Hyatt, D., Tesei, B., Fioretti, D.P. Prevalence of tick borne pathogens in horses from Italy. *J. Vet. Med. Sci.* **2013**. 75, 715–20.

Laus, F., Spaterna, A., Faillace, V., Veronesi, F., Ravagnan, S., Beribé, F., Cerquetella, M., Meligrana, M., Tesei, B. Clinical investigation on *Theileria equi* and *Babesia caballi* infections in Italian donkeys. *BMC Vet. Res.* **2015.** 11, 100.

Machado, R.Z., Toledo, C.Z.P., Teixeira, M.C. a, André, M.R., Freschi, C.R., Sampaio, P.H. Molecular and serological detection of *Theileria equi* and *Babesia caballi* in donkeys (Equus asinus) in Brazil. *Vet. Parasitol.* **2012.** 186, 461–5.

Moretti, A., Mangili, V., Salvatori, R., Maresca, C., Scoccia, E., Torina, A., Moretta, I., Gabrielli, S., Tampieri, M.P., Pietrobelli, M. Prevalence and diagnosis of Babesia and Theileria infections in horses in Italy: A preliminary study. *Vet. J.* **2010.** 184, 346–350.

Nagore, D., García-Sanmartín, J., García-Pérez, A.L., Juste, R. a., Hurtado, A. Detection and identification of equine Theileria and Babesia species by reverse line blotting: Epidemiological survey and phylogenetic analysis. *Vet. Parasitol*. **2004**. 123, 41–54.

Nardoni, S., Sgorbini, M., Gugliucci, B., Merildi, V., Cammà, C., Franceschi, A., Corazza, M., Mancianti, F. Preliminary study on the variation of clinical and parasitological features in a group of Amaiata donkeys infected by *Theileria equi*. *Parassitologia*, **2007**. 49, suppl 1, 97.

Oduori, D.O., Onyango, S.C., Kimari, J.N., MacLeod, E.T. A field survey for the seroprevalence of *Theileria equi* and *Babesia caballi* in donkeys from Nuu Division, Kenya. *Ticks Tick. Borne. Dis.* **2015**. 6, 683–688.

OIE Terrestrial Animal Health Code Twentieth edition, **2011.**

Pfäffle, M., Littwin, N., Muders, S. V., Petney, T.N. The ecology of tick-borne diseases. *Int. J. Parasitol*. **2013.** 43, 1059–1077.

Piantedosi, D., D'Alessio, N., Di Loria, a, Di Prisco, F., Mariani, U., Neola, B., Santoro, M., Montagnaro, S., Capelli, G., Veneziano, V. Seroprevalence and risk factors associated with *Babesia caballi* and *Theileria equi* infections in donkeys from Southern Italy. *Vet. J*. **2014.** 202, 578–82.

Scoles G., Ueti M.W. Vector ecology of equine piroplasmosis. *Annu. Rev. Entomol.* **2015**. 60, 561-580.

Sevinc, F., Maden, M., Kumas, C., Sevinc, M., Ekici, O. A comparative study on the prevalence of *Theileria equi* and *Babesia caballi* infections in horse sub-populations in Turkey. *Vet. Parasitol.* **2008** .156, 173–177.

Shchuchinova LD, Kozlova IV, Zlobin VI: Influence of altitude on tick-borne encephalitis infection risk in the natural foci of the Altai Republic, Southern Siberia. *Ticks Tick Borne Dis* **2015,** 6:322–329.

Shkap, V., Cohen, I., Leibovitz, B., Savitsky, Pipano, E., Avni, G., Shofer, S., Giger, U., Kappmeyer, L., Knowles. Seroprevalence of *Babesia equi* among horses in Israel using competitive inhibition ELISA and IFA assays. *Vet. Parasitol*. **1998.** 76, 251–259.

Steinman, A., Zimmerman, T., Klement, E., Lensky, I.M., Berlin, D., Gottlieb, Y., Baneth, G. Demographic and environmental risk factors for infection by *Theileria equi* in 590 horses in Israel. *Vet. Parasitol*. **2012**. 187, 558–562.

Sumbria, D., Singla, L.D., Sharma, A., Bal, M.S., Randhawa, C.S. Molecular survey in relation to risk factors and haemato-biochemical alteration in *Theileria equi* infection of equines in Punjab Province, *India. Vet. Parasitol. Reg. Stud. Reports*. **2017**. 8, 43–50.

Thrusfield, M. Veterinary Epidemiology, 3rd Edition. Wiley-Blackwell, USA, **2007.** 624 pp.

Vanwambeke SO, Sumilo D, Bormane A, Lambin EF, Randolph SE: Landscape predictors of tick-

borne encephalitis in Latvia: land cover, land use, and land ownership. *Vector Borne Zoonotic Dis.* **2010**, 10:497–506.

Veronesi, F., Laus, F., Moretta, I., Fioretti, D.P., Spaterna, A., Tesei, B., Moretti, A. Prevalenza di *Babesia caballi* e *Theileria equi* in cavalli di razza tolfetana. *Ippologia.* **2010.** 21, 3–9.

Veronesi, F., Morganti, G., Ravagnan, S., Laus, F., Spaterna, A., Diaferia, M., Moretti, A., Fioretti, D.P., Capelli, G. Molecular and serological detection of tick-borne pathogens in donkeys (Equus asinus) in Italy. *Vet. Microbiol*. **2014**. 173, 348–54.

Vranova, M., Alloggio, I., Qablan, M., Vyskocil, M., Baumeisterova, A., Sloboda, M., Putnova, L., Vrtkova, I., Modry, D., Horin, P. Genetic diversity of the class II major histocompatibility DRA locus in European, Asiatic and African domestic donkeys. *Infect. Genet. Evol*. **2011** .11, 1136–1141.

Walker, A.R., Bouattour, A., Camicas, J.L., Estrada-Peña, A., Horak, I.G., Latif, A., Pegram, R.G., Preston, P.M. Ticks of Domestic Animals in Africa. A Guide to Identification of Species**. 2003.** *Bioscience Reports*, UK.

Zanet S., Bassano, M., Trisciuoglio, A., Taricco, I., Ferroglio, E. Horses infected by Piroplasms different from *Babesia caballi* and *Theileria equi*: species identification and risk factors analysis in Italy. *Vet. Parasitol.* **2017.**

STUDY 2.3

Sequence heterogeneity in the 18S rDNA gene within *Theileria equi* and *Babesia caballi* and in the equi merozoite antigen gene (ema-1) of *Theileria equi* from horses, donkeys and ticks in Central-Southern Italy.

Abstract

One hundred blood samples of symptomatic and asymptomatic equids from Central-Southern Italy were chosen for their strong positive results in real time PCR targeting the 18S gene or because of a their discordant results between ELISA and PCR test. A nested PCR protocol amplifying the hypervariable V4 region of the 18rDNA to typify the piroplasms and an end point PCR targeting EMA1 (Equi merozoite antigen gene) were performed. Also twenty-six free living ticks were tested with the EMA PCR. All positive PCR products were sequenced. Sequences showed a minimum of 98% "query coverage" and 98% homology with sequences deposited in GenBank. Phylogenetic analyses using genetic distance and homology confirmed that sequences of both parasites could be divided into 3 groups (for *B.caballi* and *T. equi*) using the V4 PCR. Also the EMA phylogenetic analysis showed the presence of three groups. Some sequences were selected to build phylogenetic trees and differences among sequences and groups were studied.

The relations among the phylogenetic groups and some variables such as the presence of symptomatology, discordance between serological test and PCR, coinfection (positives to both real time PCR) and lack or faint positivity to the EMA PCR (for *Theileria* sequences) were evaluated by Chi square or Fisher's exact test. For *Theileria* differences among groups related to the presence of symptoms, discordance between serological test and PCR and discordant EMA PCR results were significant. No variables were significant in the *B.caballi* analysis.

Piroplasms groups are apparently independent from the geographic distribution, as all have been reported by different authors worldwide. The biological significance of the correlation among genetic groups and the clinical and serological patterns require further investigations.

Keywords:

Equine piroplasmosis, phylogenetic analyses, 18S rRNA gene, EMA-1

Introduction

The use of PCR tests can overcome the difficulties in detection in cases of low parasitaemia in chronic infections and could also allow the identification of genetic heterogeneity. Different genes have been used as targets in piroplams detection such as EMA (Battsetseg *et al*., 2002), *β*-tubulin (Cacciò *et al.*, 2000) and 18S rDNA (Nagore *et al.,* 2004).

Merozoite surface proteins have an essential role in the recognition, attachment and penetration of the parasites in the erythrocytes. Equi merozoite antigen-1 (EMA-1) is a 34 kDa immunodominant protein used in the cELISA diagnostic tests and although it was originally described as a single copy gene it has been confirmed that 10 genes are present in this family (Kappmeyer *et al.*, 2012). Due to their surface location, they are likely to be under intense selection pressure, and sequence heterogeneity in the genes encoding these proteins is expected to happen (Allsopp & Allsopp, 2006). The EMA-1 heterogeneity has been evidenced in studies conducted in South Africa by Bhoora *et al*. (2010b).

The small subunit ribosomal RNA (18S rDNA) gene is a highly conserved region in the genome, analogous to the 16S in prokaryotes. Due to its high levels of conservation, its low substitution rate and its multiple copies, the 18S rDNA gene represents the most suitable genetic marker for diagnosis, characterization, taxonomic classification and phylogenetic studies of piroplasmids (Allsopp & Allsopp, 2006). Several 18S rDNA-based phylogenetic analyses have been performed by different authors: Kouam *et al*. (2010) in Greece, Veronesi *et al.* (2014) in Italy. Bhoora *et al.* (2009) in South Africa; Salim *et al*. (2010) in Sudan; Qablan *et al*. (2013) in Jordan, Seo *et al*. (2013) in Korea, and Hall *et al*. (2013) in USA. These studies indicated a remarkable degree of variation within and among *B. caballi* and *T. equi* such as those of Spanish sequences authors (Nagore *et al*., 2004; Criado Fornelio *et al*., 2006).

Material and methods

One hundred blood samples from Central-Southern Italian equidae collected between 2013 and 2014, 77 horses (35 symptomatic and 42 asymptomatic) and 23 donkeys (2 symptomatic and 21 asymptomatic) were chosen for their strong positive results in real time PCR (Ct <24) targeting the 18S gene or because of a their discordant results between ELISA and

PCR test. Included in the symptomatic horses samples, there were two mares (study 2.3) that aborted and their foetus and foetal remains also were sampled.

In order to typify the piroplasms a nested PCR protocol amplifying the hypervariable V4 region of the 18S rDNA was performed.

To investigate the presence of piroplasms in the vectors 26 adult free living ticks were collected by drag-flag method in the Monti Aurunci Nature Park, placed between two Latium provinces, range coordinates 41°21'N 13°40'E, where many serological and PCR positive animals have been detected. Tick identification was performed according to taxonomic keys described by Walker *et al.* (2003).

An end point PCR targeting the merozoite antigen 1 on 97 equidae samples and on the ticks samples (18S PCRs hybridised the tick genome) was carried out.

Samples

Blood samples were collected from the jugular vein and placed into serum and EDTA tubes. Sera were obtained by centrifugation for 10 minutes at 358 g; then stored at –20°C and thawed at +37°C immediately before testing. Foetal remains and organs were stored at -20°C until processed.

Ticks were conserved in 70% ethanol solution at −80°C.

Serological tests

Two commercial competitive enzyme-linked immunosorbent assays (cELISA): *Babesia equi* Antibody test kit VMRD® and *Babesia caballi* Antibody test kit VMRD® were performed according to manufacturers instructions.

DNA extraction from blood and organs

DNA extraction was performed using the QIAamp DNA Blood Mini kit (Qiagen, GmbH, Hilden, Germany) following the instructions of the manufactures. The DNA was eluted in 200 µl buffer AE and stored at –20°C.

To extract DNA from the foetal organs, the same protocol was used adding one step before, 100 milligrams of organ in 600 µl of 1X PBS were placed in a FastPrep tub and then homogenize in a FastPrep FP120 Cell Disrupter (Thermo Electron) twice at 12000 rpm 5 minutes. Supernatant was collected and use to assess DNA extraction.

DNA extraction from ticks

DNA extraction from adult ticks was performed using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) following a modified protocol for tissues with the following alterations: after 3 hours incubation in 180 µl buffer ATL at 56°C with 20 µl proteinase K, ticks were homogenized mechanically and incubated 2 hours in buffer AL at 70°C. The DNA was eluted in 100 µl of buffer AE. DNA yield was determined with a spectrophotometer (Eppendorf BioPhotometer, Eppendorf AG, Hamburg).

Real time T.equi *and real time* B. caballi

Real time PCR 18S rRNA *B. caballi* amplified a 95bp fragment in the V4 Hypervariable region of 18S rRNA gene of *B. caballi* and the forward primer (Bc-18SF402) and the probe (TaqMan MGB® probe (FAM-MGB), Bc-18SP) were specific for *B. caballi* but the reverse primer (Bc-18SR496) was not (Bhoora *et al.*, 2010a).

The real time 18S rRNA *T.equi* amplified an 81bp fragment in the V4 hypervariable region of 18S rRNA gene *T. equi* and the forward and reverse primers (Be18SF and Be18SR) and the probe (a TaqMan probe (VIC-TAMRA, Be 18SP) were specific for *T. equi* (Kim *et al*., 2008).

The positive controls were constituted by plasmid vectors pCRII-TOPO TA (TOPO TA Cloning® Invitrogen, Carlsbad, CA, USA) in which the targets of the real time PCR *T. equi* and *B. caballi* have been cloned.

EMA end point PCR

The target for EMA end point PCR was a 268 bp fragment internal at *Theileria equi* merozoite antigen 1 (EMA-1) gene coding for a major parasite surface antigen and the primers used were EMA-5/EMA-6 (Battsetseg *et al.*, 2002).

Typifying nested PCR Protocol

Another PCR test was carried out to typify the piroplasms; it amplifies the hypervariable V4 region of the 18rRNA gene of the genera *Theileria* and *Babesia* with a nested PCR. This protocol amplifies products of approximately 430bp and 390bp for *Theileria* and *Babesia* species respectively; primers RLB F1 RLB R1, RLB F2 RLB R2, (Nagore *et al.*, 2004). The thermal profile was optimised, in order to avoid nonspecific reactions, changing the annealing temperature from 51°C indicated in literature to 54°C.

In all protocols AmpliTaq Gold® DNA Polymerase (A. Biosystems, Life Technologies, Austin, Tx, USA) to prepare the master mix and the GeneAmp® PCR System 9700 (A. Biosystems, Foster City, CA, USA) were used.

PCR products were visualized after electrophoresis in a 1.5% Tris-Boric Acid-EDTA agarose gel and stained with GelRed 10,000X (Biotium, Hayward, C, USA).

All the products were recovered from agarose gel using the QIAquick® PCR Purification kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions and sequenced using the PCR primers of second PCR for V4 PCR and EMA primers with the BigDye Terminator Cycle Sequencing Ready Reaction kit, version 3.1 (PerkinElmer, A. Biosystems, Foster City, CA, USA) in an automated sequencer (3500 Genetic Analyzer, A. Biosystems, Foster City, CA, USA). The nucleotide sequences obtained were analysed using the Genetic Analyzer Sequencing v5.4 (A. Biosystems, Foster City, CA, USA).

Phylogenetic analysis

Sequences were assembled and aligned using Geneious 9.1.3. Sequence homology was performed using the Basic Local Alignment Search Tool (BLAST) by comparing them to sequences of equine piroplasms in NCBI GenBank (http://www.ncbi.nlm.nih.gov).

Phylogenetic analyses using distance and homology were used to find out the presence of different groups.

Forty-nine selected *Theileria equi* consensus sequences, six *Babesia caballi* consensus sequences from products of the nested PCR and some Genbank registered sequences (for *T.equi* Z15105, AY534882 and AB515315 and for *B.caballi* Z15104, AY534883, and EU642514) were chosen to build phylogenetic trees using Geneious 9.1.3. Tamura-Nei genetic distance model and neighbor-joining method using as outgroup the sequence DQ439543.1 (*Hepatozoon canis* isolate1).

Consensus sequences from EMA PCR products were also analysed; 15 sequences from equids, 13 tick sequences and some Genbank registered sequences (AAC38827, BAA96134, BAA32979) were chosen to build phylogenetic trees using Geneious 9.1.3, same settings as described above using as outgroup the sequence AAX55745.1 (*Plasmodium falciparum*).

Statistical analysis

The relations between explanatory variables and the phylogenetic groups were

evaluated by Chi square or Fisher's exact test. Selected variables were: presence of symptomatology, discordance between serological test and PCR, coinfection (positives to both real time PCR) and lack or faint positivity to the EMA PCR (for *Theileria* sequences). A p value <0.05 was considered statistically significant. SAS 9.4 software for Windows was used for all statistical analyses.

Results

Sequenced products of the nested PCR conducted on the 100 EP positive samples belonged to strains of *Theileria equi* (94) and *Babesia caballi* (9). Sequences showed a minimum of 98% “query coverage” and at least 98% of homology with sequences deposited in GenBank.

Phylogenetic analyses using distance and homology showed that both *T. equi* and *B.caballi* sequences could be divided into three different groups. Group 1 contains sequences homologous to the first submitted piroplasms sequences (Z15105, Z15104), group 2 includes “like genotypes” (AY534882, AY534883) and group 3 contains sequences with equidistant homology from groups one and two (AB515315, EU642514). Groups have been named 1, 2 and 3 in a chronological order of description, although the clades are not distributed in this order in our trees. Regarding to *Babesia*, 5 samples belonged to group 1, 2 to group 2 and 2 to group 3 (all were obtained from horse samples); for *Theileria* 41 sequences belonged to group 1, 38 to group 2 and 15 to group 3. By species, the 71 horses sequences were distributed: 40 in group 1, 26 in group 2 and 5 in group 3. In donkeys 1 belonged to group 1, 12 to group 2 and 10 in group 3.

The statistical analysis in the *Theileria* sequences showed significant differences among groups related to the presence of symptoms (60 asymptomatic and 34 symptomatic); group 1 sequences were found in symptomatic animals more than in asymptomatic animals which presented sequences of groups 2 and 3. Discordance between the serological test and PCR (32 samples) was also significant, being the group 1 sequences more present in the seronegative animals while seropositive animals presented mostly sequences of groups 2 and 3. Significant differences among groups were found related to EMA PCR results: negative or faint positivity to the end point PCR samples (42) were mostly group 2 and 3 sequences. 22 samples presented coinfection (positive to both real time protocols although not all could be sequenced), but this was not a significant factor.

The statistical analysis performed on *B*. *caballi* sequences didn't show significant values may be due to the few number of samples.

Since most of the donkeys (21/23) were asymptomatic, in order to avoid potential bias associated with collinearity, the factor species was not considered.

More details about the statistical results can be found in the Table 1.

In the first case of abortion the sequence obtained from the intracardiac blood clot foetal sample belonged to the group 1 (KU923646); the blood of the mother was not available at moment of abortion and a blood sample of the mare was provided only eight months later when she was asymptomatic: it was found a sequence belonging to group 2 (KU923628). In the second abortion case a sequence belonging to group 1 (KU923625) was detected in the blood of the mother; a second blood sample was obtained after five months (when the mare was in asymptomatic phase) and a sequence belonging to group 2 was evidenced (KU923626). In both abortion cases the second blood mares samples were negative to the end point PCR EMA. In the cELISA tests of these sera from second blood samples, the first mare resulted seropositive but the second one was seronegative.

Forty-nine selected *Theileria equi* consensus sequences as well as three Genbank reference sequences (Z15105, AY534882 and AB515315) were chosen for sequence alignment and phylogenetic tree construction (Figure 1). Alignment results allowed to point out a 351 bp region (from nucleotide 521 to nucleotide 868 internal to V4 18S rRNA accession number Z15105) that had a 92% overall identity among sequences. This percentage increased beyond the 99% when sequences of each group were considered separately: group 1 sequences share a sequence identity of 99.8%, sequences belonging to group 2 (which appears to be subdivided in the tree) share a sequence identity of 99.5% and the sequences belonging to group 3 show a sequence identity among them of 99.1%. More details about *T. equi* V4 sequences groups are available in the Figure 2.

Table 1: Results of the statistical analyses for *T. equi* and *B. caballi.* P value ≤ 0.05 was considered significant.

V4 *Theileria equi*					
Variables	**Category**	**Group 1**	**Group 2**	**Group 3**	**P**
Symptoms N=94	Asymptomatic Symptomatic	11 30	36 2	13 2	<0.0001
ELISA test N=94	Negative Positive	24 16	5 33	3 11	<0.0001
EMA PCR N=91	Faint/negative Positive	10 28	23 15	9 6	0.0057
Coinfection N=94	No Yes	33 8	26 12	13 2	0.2710

V4 *Babesia caballi*					
Variables	**Category**	**Group 1**	**Group 2**	**Group 3**	**P**
Symptoms N=9	Asymptomatic Symptomatic	3 2	2 0	1 1	1.000
ELISA test N=9	Negative Positive	4 1	0 2	2 0	0.1667
Coinfection N=9	No Yes	1 4	1 1	1 1	1.000

The following sequences have been submitted to GenBank for *T.equi* V4:

Group 1: KU923625, KU923627, KU923638, KU923639, KU923640, KU923641, KU923642, KU923643, KU923644, KU923645, KU923646, KU923647, KU923648, KU923649, KU923650, KU923651, KU923652, KU923653, KU923654, KU923655.

Group 2: KU923615, KU923616, KU923617, KU923618, KU923619, KU923620, KU923621, KU923622, KU923623, KU923624, KU923626, KU923628, KU923656, KU923657, KU923658, KU923659, KU923660, KU923661, KU923662, KU923663.

Group 3: KU923629, KU923630, KU923631, KU923632, KU923633, KU923634, KU923635, KU923636, KU923637.

Six *Babesia caballi* consensus sequences as well as three Genbank reference sequences (Z15104, AY534883, and EU642514) were chosen for sequence alignment and phylogenetic tree construction (Figure3). Alignment results allowed to point out a 299 bp region (from nucleotide 473 to nucleotide 770 internal to V4 18S rRNA accession number Z15104 that had a 96% overall identity among sequences. This percentage increased beyond the 99% when sequences of each group were considered separately: group 1 sequences share a sequence identity of 100%, sequences belonging to group 2 share a sequence identity of 99.7% and the sequences belonging to group 3 show a sequence identity among them of 99.8%. More details about *B. caballi* sequences are available at Figure 4.

Figure 1: Rooted cladogram calculated using maximum-parsimony and bootstrap estimates from 100 replicates of the V4 hypervaryable region of 18S rRNA gene of *T. equi* and as an outgroup *Hepatozoon canis* isolate Venezuela-1 (accession number: DQ439543.1). Bootstrap values are indicated on the nodes of the tree and the scale for substitutions per site are provided.

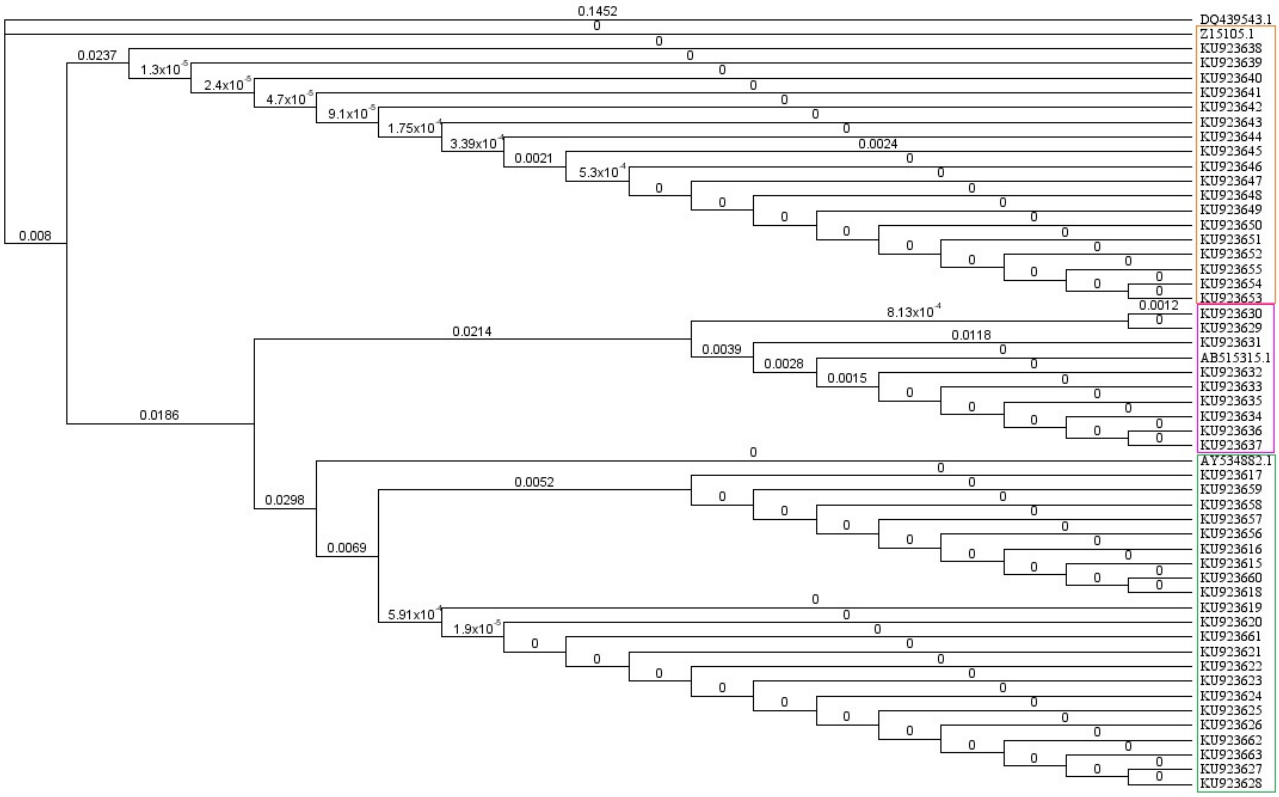
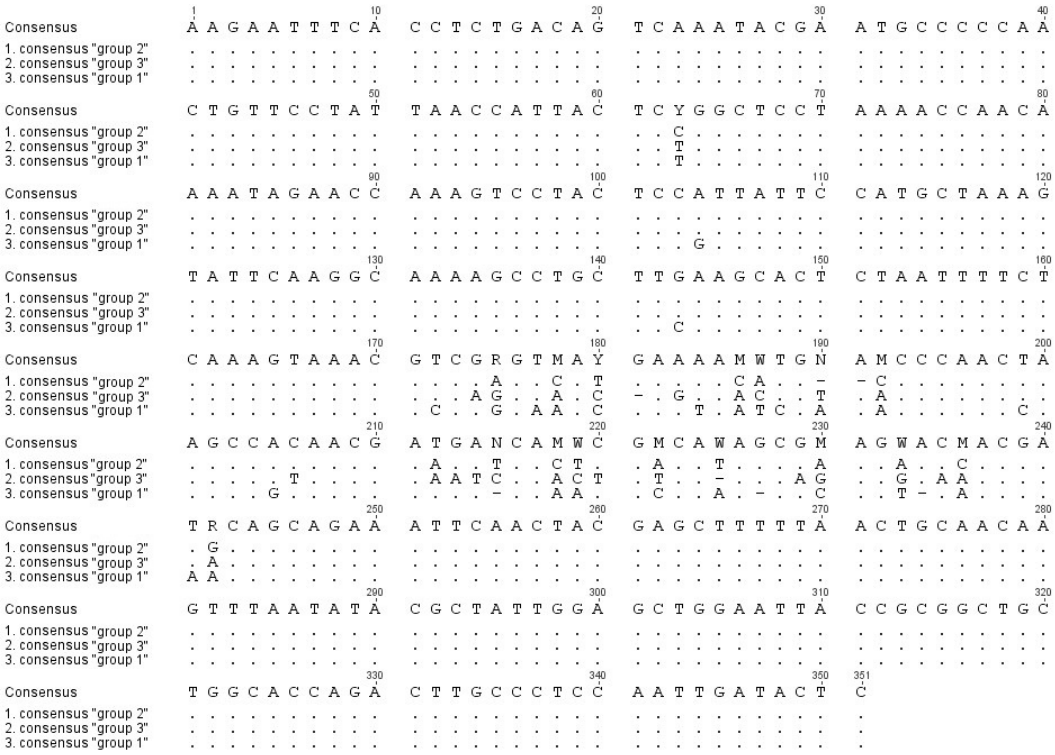


Figure 2: Nucleotide alignment of the V4 hypervariable region of the 18S rDNA gene of the *T. equi* consensus group sequence examined in this study.



Submitted sequences to GenBank for *B.caballi* V4

Group 1: KU923669, KU923668, KU923667

Group 2: KU923664

Group 3: KU923665, KU923666

Among the sequences used to construct the trees, three of them came from co-infections samples *(B. caballi* and *T.equi* sequenced). In detail in two of them, *T.equi* group 2 strains were evidenced with a *B. caballi* strain of group 1 and group 2 (in both cases it was the second sampling of the abortion mares in the asymptomatic phase at a distance of months from abortion) in the third co-infection sample, the *T. equi* strain belonged to group 3 while the strain of *B. caballi* belonged to group 1.

Figure 3**:** Rooted cladogram calculed using maximum-parsimony and bootstrap estimates from 100 replicates of the V4 hypervariable region of 18S rRNA gene of *B. caballi* and as an outgroup *Hepatozoon canis* isolate Venezuela-1 (accession number: DQ439543.1). Bootstrap values are indicated on the nodes of the tree and the scale for substitutions per site are provided.

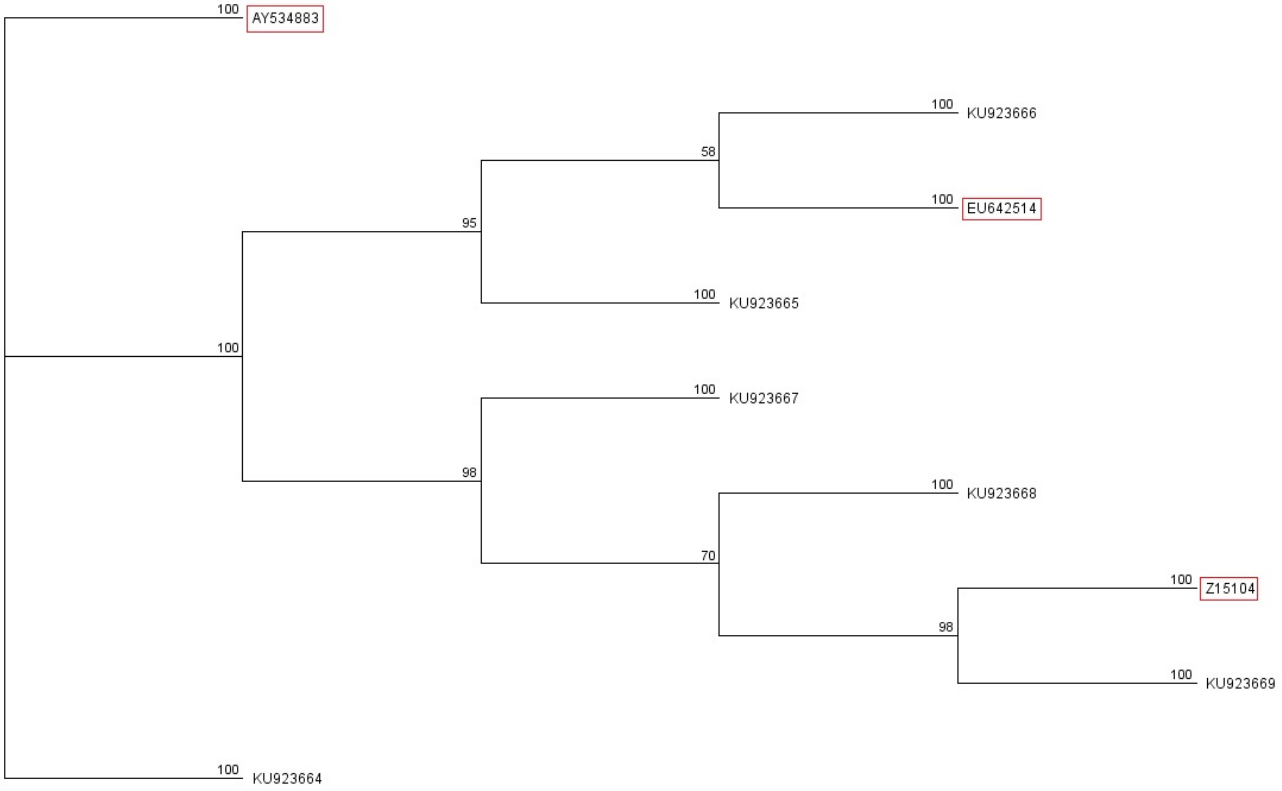
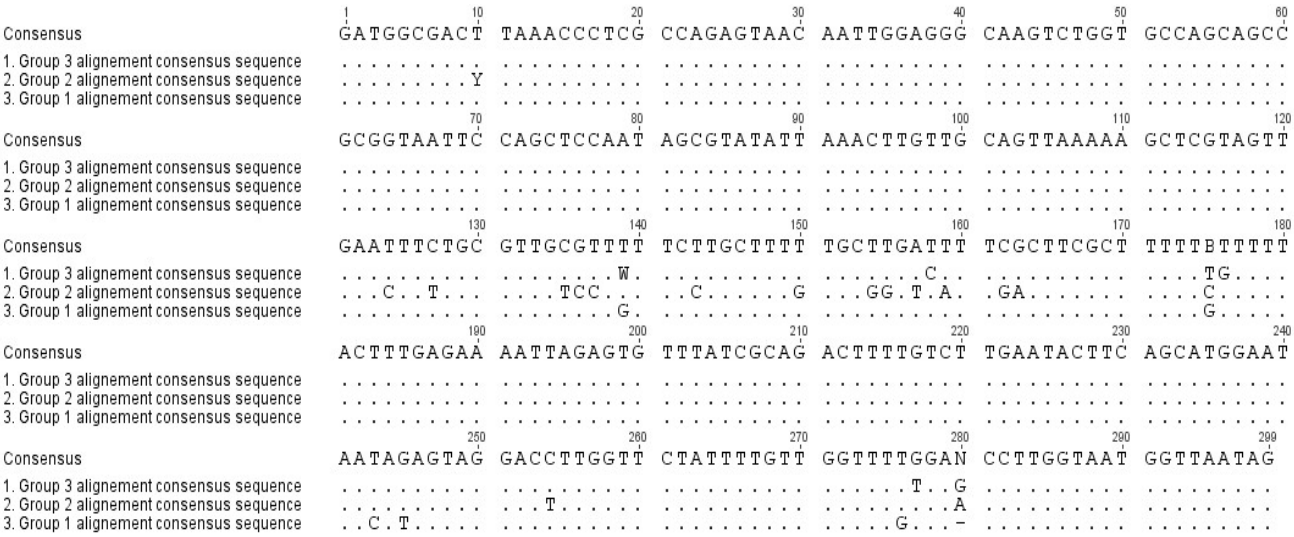


Figure 4: Nucleotide alignment of the V4 hypervariable region of the 18S rDNA gene of the *B. caballi* consensus group sequence examined in this study.



The ticks found were identified as *Dermacentor marginatus*, 15 of 26 ticks were positive to PCR EMA: 13 sequences of them along with 15 sequences obtained from EMA PCR positive horses (all symptomatic except 1 animal) and some Genbank reference sequences (AAC38827, BAA96134, BAA32979) were used for the construction of phylogenetic trees as described by other authors (Bhoora *et al*., 2010b). In their study, sequences could be divided into 3 different groups, in our tree the 3 groups can be evidenced using the mentioned reference sequences but among our sequences there are no representative ones of the group 3, our sequences are distributed just in the group 1 where 26 sequences were found (all ticks and 13 horses, including abortion samples KU923592, and the symptomatic mare KU923606) and in group 2 where two horse sequences were individuated.

The 28 consensus sequences chosen for the construction of the phylogenetic tree (Figure 5) using the portion of the gene coding for the antigen of the merozoite 1 *Theileria equi* were analysed and alignment results allowed to point out a 69 amino acid region (from amino acid 43 to amino acid 111 internal to the EMA1 gene region accession number AAC38827). The homology between the sequences of the group 2 is 100% and among those in group 1 is 99.8%. The homology between group 1 and 2 is 99%. More information about the sequences is available in Figure 6.

It was found no correspondence among the V4 groups and the groups of EMA.

EMA recorded sequences submitted to GenBank:

Group 1: KU923588, KU923589, KU923590, KU923591, KU923592, KU923593, KU923594, KU923595, KU923596, KU923597, KU923598, KU923599, KU923600, KU923602, KU923603, KU923604, KU923605, KU923606, KU923607, KU923608, KU923609, KU923610, KU923611, KU923612, KU923613, KU923614

Group 2: KU923587, KU923601

Discussion

Several piroplasms phylogenetic divisions based on the 18S rRNA have been reported all over the world. Two genotypes within *Babesia* and *Theileria* were first described in Spain by Nagore *et al.* (2004), named “like forms”. In South Africa, Bhoora *et al*. (2009) have described 3 heterogeneous groups for *Theileria* (Groups A, B and C) in horses and zebras and 2 for *Babesia* (Groups A and B; B group is subdivided), Kouam *et al*. (2010) also have reported

Babesia and *Theileria* "*like*" forms in Greece. Salim *et al*. (2010) described four *T. equi* groups in Sudan samples, Qablan *et al.* (2013) observed five *Theileria equi* groups in Jordan; Hall *et al.* (2013) observed four *T. equi* groups in USA samples and Veronesi *et al.* (2014) observed four *T. equi* groups in Italian donkeys. In our study three groups have been determined which we have called them group 1, group 2 and group 3.

T. equi group 1 was found in symptomatic animals and this matches with Hall *et al.* (2013), whose outbreak samples are most located in the same group. However Nagore *et al.* (2004) found the *T. equi* "like form" in a symptomatic animal. Groups 2 and 3 seem to be more prevalent in asymptomatic infections.

Regarding the abortions cases, it was surprising that in both mares which showed group 1 sequences during the symptomatic process (abortion) after some months, showed group 2 sequences in the second samples collected and a lack of positivity to EMA PCR; for one of them also a lack of positivity to the ELISA test was also recorded. This observation could be explained either by a reinfection with another strain or by the presence of coexisting forms that we couldn't detect at the initial stage of infection. Nagore *et al.* (2004) and Kouam *et al.* (2010) reported the presence of coexisting *T. equi* groups by reverse line blot hybridisation assay but our PCR just can evidence the predominant form in case of coinfection (*B. caballi* and *T. equi*) or coexisting forms (groups 1, 2 and 3); in the electropherogram results of the mares there was no evidence of mixed sequences or groups, so it seemed the mares didn't show coexisting groups when tested.

Discordances between *T. equi* ELISA and PCR results are more frequent in group 1, so in symptomatic animals, this could be due to the fact that animals were tested during acute infections and they have not developed specific antibodies; although in many cases our strains of groups 2 (one of the abortion mares) and 3 came from seronegative animals. A horse in the Hall *et al*. (2013) study showed faint positivity in the serological survey and its *T. equi* sequence belonged to group 3. This could be due to a lack of expression of the EMA1 gene or in alternative to an escape mutant which could cause negative results in the serological assay, these negative results should be controlled by means of more sampling at time distances to check the evolution as *T. equi* antibodies are lifelong. The antigen protein EMA 1 could be exposed to a high selection pressure which could lead to the presence of a higher variability in order to evade the immune system which could support the theory of the escape mutant.

Cases of faint or negativity results to EMA1 end point PCR are prevalent in groups 2 and 3, and this agrees with the observation of the same animal described before by Hall *et al.* (2013) whose *T. equi* strain was group 3 and it showed not only faint seropositivity but was negative to the EMA PCR.

Three groups were evidenced for *B.caballi*, Bhoora *et al*. (2009) found two but one was subdivided; just nine sequences could be characterised on 30 real time positive samples, this could due to the low parasitaemia present in *B.caballi* infections (de Waal, 1992), statistical analyses were not significant because of the low number or samples, so no relationship between symptomatology or ELISA results could be stabilised.

Negative EMA1 PCR results could be related to a problem with the detection limit in animals showing low parasitaemia or by the existence of parasites variants, or presence of mechanisms of immune evasion. These mecanisms have been described in other protozoa in which genetic mechanisms have been observed or postulated to control antigenic variation such as segmental gene conversion, transcriptional switching, alterations of chromosomal structure associated with transcriptional control and recombination during sexual reproduction (Allred, 2001), although these hypotheses have not been supported by other authors like Kappmeyer *et al.* (2012) who don't find evidence that the EMA1 genes undergo recombination or dynamic sequence variation, as it could be expected in genes involved in immune evasion. Therefore Ketter-Ratzon *et al.* (2017) suggest EMA-1 gene was not a good gene for the evaluation of heterogeneity in *T. equi*.

Regarding the EMA1 phylogenetic analysis, using the reference sequences previously mentioned, three groups were observed as Bhoora *et al.* (2010b) described in their study, although no group 3 samples were obtained by us. It is interesting to note that our group 1 sequences seem to be older than the reference clade sequence used AAC38827 as the reference sequence shows two more mutations which are not present in our sequences.

Figure 5: Phylogenetic *tree T. equi* EMA

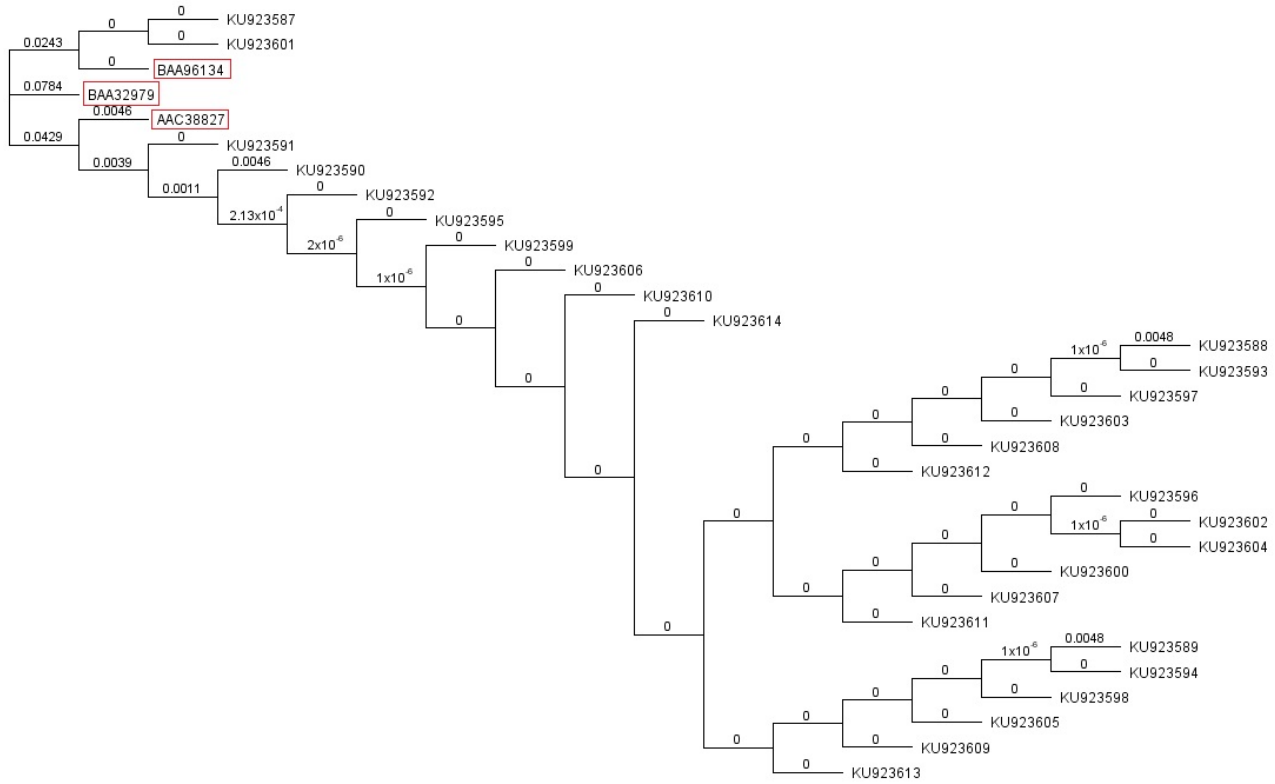
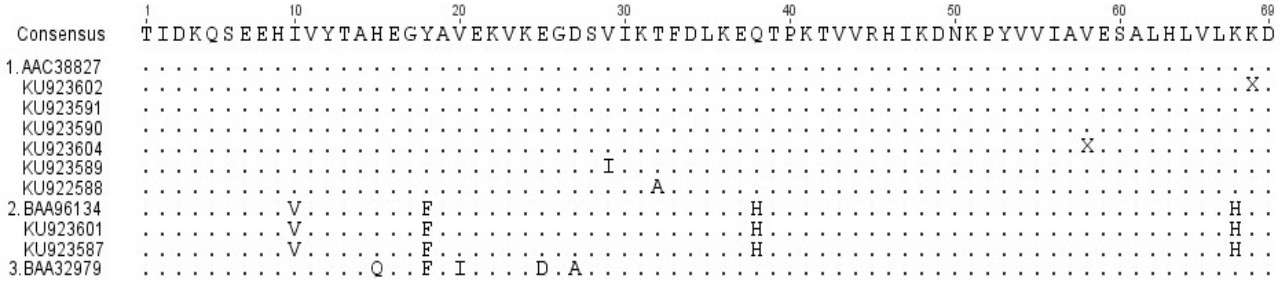


Figure 6: Nucleotide alignment of EMA 1 gene of the *T .equi* consensus group sequence examined in this study.



In our study no relation among V4 classification and EMA classification could be established as authors described. There is no correspondence between EMA group and seronegativity as samples of group 1 and 2 were Ealisa negative so it is impossible to determine if it is due to the fact that animals were symptomatic, so may be there were acute cases in which antibodies were no yet present, more than differences in expression of EMA1 gene.

Conclusion

Three principal clades for *B. caballi* and *T. equi* have been described in different countries worldwide so clustering seems to be independent from geographical distribution. Also in our study three clades have been observed for both *T. equi* and *B.caballi* using the 18S

rDNA, for *T. equi* using the target EMA1 gene, also three groups could be evidenced, but no relation among the V4 classification and EMA classification has been established, also because some group 2 and 3 showed faint or lack of positivity so, group determination was not possible.

In the present study, very interesting results have been observed related to the parasite group changes detected in two mares as shown by the DNA sequencing of the hypervariable V4 region: possible explanations for these results are either a co-infection sustained by two different *T. equi* groups or a second infection occurred between the two samples collection. Also the negative results to the EMA1 PCR and the lack of EMA1 antigen detection (ELISA negative results) in one of the mares second sample could be explained by an adaptation form to evade the immune system of the host.

V4 18 S rDNA is the best target to detect infections in low parasitaemia cases: it is much conserved, it is a multicopies target and it is not exposed to a selective pressure as EMA1 antigen.

No influence of host species (donkeys, horses and ticks) was proven for the sequences distribution within the different classification groups (V4 for *B. caballi* and *T. equi* and EMA1 for *T. equi*).

It would be very interesting to elaborate a serial sampling design to evaluate changes in parasitic populations and to develop new techniques to determine the presence of the different groups and parasites so it could be studied the relationships of each one and other variables such as negative serological results and pathogenicity.

Conflict of interests

The author declares no conflict of interests.

Acknowledgements

This study was developed with funds from the IZSLT 16/11 project (provided by the Italian Ministry of Health).

Author contribution

Blood collection from some animals, tick collection, DNA extraction, PCR performance, DNA purification, sequence alignments, partial examination of sequences, phylogenetic tree construction and manuscript redaction.

References

Allsopp, M.T.E.P., Allsopp, B.A., Molecular sequence evidence for the reclassification of some *Babesia* species. Annals of the New York Academy of Sciences **2006**.1081, 509–517.

Allred, D. R. Molecular technology and antigenic variation among intraerythrocytic hemoparasites: Do we see reality? *Vet. Parasitol.* **2001**, *101*, 261–274.2.

Battsetseg, B.; Lucero, S.; Xuan, X.; Claveria, F. G.; Inoue, N.; Alhassan, A.; Kanno, T.; Igarashi, I.; Nagasawa, H.; Mikami, T.; Fujisaki, K. Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. *Vet. Parasitol.* **2002**, *107*, 351–357.3.

Bhoora, R.; Franssen, L.; Oosthuizen, M. C.; Guthrie, A. J.; Zweygarth, E.; Penzhorn, B. L.; Jongejan, F.; Collins, N. E. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. *Vet. Parasitol.* **2009**, *159*, 112–120.4.

Bhoora, R.; Quan, M.; Franssen, L.; Butler, C. M.; Van der Kolk, J. H.; Guthrie, A. J.; Zweygarth, E.; Jongejan, F.; Collins, N. E. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. *Vet. Parasitol.* **2010a**, *168*, 201–211.5.

Bhoora, R.; Quan, M.; Matjila, P. T.; Zweygarth, E.; Guthrie, A. J.; Collins, N. E. Sequence heterogeneity in the equi merozoite antigen gene (ema-1) of *Theileria equi* and development of an ema-1-specific TaqMan MGB assay for the detection of *T. equi*. *Vet. Parasitol.* **2010b**, *172*, 33–45.6.

Cacciò, S.; Cammà, C.; Onuma, M.; Severini, C. The β-tubulin gene of *Babesia* and *Theileria* parasites is an informative marker for species discrimination. *Int. J. Parasitol.* **2000**, *30*, 1181–1185.7.

Criado, A.; Martinez, J.; Buling, a.; Barba, J. C.; Merino, S.; Jefferies, R.; Irwin, P. J. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. *Vet. Parasitol.* **2006**, *142*, 238–247.8.

de Waal, D. T. Equine piroplasmosis: a review. *Br. Vet. J.* **1992**, *148*, 6–14.9.

Hall, C. M.; Busch, J. D.; Scoles, G. a; Palma-Cagle, K. a; Ueti, M. W.; Kappmeyer, L. S.; Wagner, D. M. Genetic characterization of *Theileria equi* infecting horses in North America: evidence for a limited source of U.S. introductions. *Parasit. Vectors* **2013**, *6*, 35.10.

Kappmeyer, L. S.; Thiagarajan, M.; Herndon, D. R.; Ramsay, J. D.; Caler, E.; Djikeng, A.; Gillespie, J. J.; Lau, A. O.; Roalson, E. H.; Silva, J. C.; Silva, M. G.; Suarez, C. E.; Ueti, M. W.; Nene, V. M.; Mealey, R. H.; Knowles, D. P.; Brayton, K. a Comparative genomic analysis and phylogenetic position of *Theileria equi. BMC Genomics* **2012**, *13*, 603.11.

Ketter-Ratzon, D.; Tirosh-levy, S.; Nachum-biala, Y.; Saar, T.; Qura, L.; Zivotofsky, D.; Abdeen, Z.; Baneth, G.; Steinman, A. Accepted. *Ticks Tick. Borne. Dis.* **2017**.

Kim, C. M.; Blanco, L. B. C.; Alhassan, A.; Iseki, H.; Yokoyama, N.; Xuan, X.; Igarashi, I. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. *Vet. Parasitol.* **2008**, *151*, 158–163.13.

Kouam, M. K.; Kantzoura, V.; Masuoka, P. M.; Gajadhar, A. a.; Theodoropoulos, G. Genetic diversity of equine piroplasms in Greece with a note on speciation within *Theileria* genotypes (*T. equi and T. equi-like*). *Infect. Genet. Evol.* **2010**, *10*, 963–968.14.

Nagore, D.; García-Sanmartín, J.; García-Pérez, A. L.; Juste, R. a.; Hurtado, A. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: Epidemiological survey and phylogenetic analysis. *Vet. Parasitol.* **2004**, *123*, 41–54.15.

Qablan, M. a; Oborník, M.; Petrželková, K. J.; Sloboda, M.; Shudiefat, M. F.; Hořín, P.; Lukeš, J.; Modrý, D. Infections by *Babesia caballi* and *Theileria equi* in Jordanian equids: epidemiology and genetic diversity. *Parasitology* **2013**, *140*, 1096–103.16.

Salim, B.; Bakheit, M. a.; Kamau, J.; Nakamura, I.; Sugimoto, C. Nucleotide sequence heterogeneity in the small subunit ribosomal RNA gene within *Theileria equi* from horses in Sudan. *Parasitol. Res.* **2010**, *106*, 493–498.17.

Seo, M. G.; Yun, S. H.; Choi, S. K.; Cho, G. J.; Park, Y. S.; Cho, K. H.; Kwon, O. D.; Kwak, D. Molecular and phylogenetic analysis of equine piroplasms in the Republic of Korea. *Res. Vet. Sci.* **2013**, *94*, 579–583.18.

Veronesi, F.; Morganti, G.; Ravagnan, S.; Laus, F.; Spaterna, A.; Diaferia, M.; Moretti, A.; Fioretti, D. P.; Capelli, G. Molecular and serological detection of tick-borne pathogens in donkeys (Equus asinus) in Italy. *Vet. Microbiol.* **2014**, *173*, 348–54.

Walker, A.R., Bouattour, A., Camicas, J.L., Estrada-Peña, A., Horak, I.G., Latif, A., Pegram, R.G., Preston, P.M. Ticks of Domestic Animals in Africa. A Guide to Identification of Species**. 2003.** *Bioscience Reports*, UK.

STUDY 2.4

Evidence of transplacental transmission of equine piroplasms *Theileria equi* and *Babesia caballi* in two Italian breed mares

Abstract

Equine piroplasmosis is a vector borne disease caused by protozoans *Babesia caballi* and *Theileria equi*. Carrier mares may transmit the infection by transplacental transmission resulting in abortion or neonatal piroplasmosis. This event has been described for *T. equi* by several authors, but no evidence of it has been reported for *B. caballi* in Europe before. In this study transplacental transmission for both parasites has been evidenced using molecular and serological tools and microscopic examinations have also been conducted.

Introduction

Equine piroplasmosis is a tick-borne disease caused by *Babesia caballi* and *Theileria equi* that affects horses, mules, donkeys and zebras. Both parasites are transmitted by ticks of genres *Dermacentor, Rhipicehalus and Hyalomma.* This disease is globally distributed and causes subclinical and clinical infections, and death. Transplacental transmission of *Theileria equi* has been reported before whereas no evidence has been descrived for *Babesia caballi* in Europe although suspected (Sant *et al.*, 2016). In utero infections may result in abortions, stillbirths, or birth of live foals with neonatal piroplasmosis (Allsop *et al.*, 2007, Georges *et al*., 2011, Chhabra *et al*., 2012). Foals born of infected mares are naïve at birth and acquire antibodies through the colostrum intake (Kumar *et al*., 2008). Diagnostic can be performed by stained blood identification, serological tests such as complement fixation test, indirect fluorescent antibody test (IFAT), ELISA or PCR methods. Equine piroplasmosis is responsible for important economic losses to equine husbandry being a major constraint to the international movement of equines. The objective of this study was to investigate the transplacental transmission of *T. equi* and *B. caballi* in two abortion cases with a suspicion of equine piroplasmosis infection in two Italian breed mares.

Material and methods

Equine samples:

During 2013 and 2014 two cases of abortion suspected to be caused by piroplams in two Italian breed mares were reported to the National Reference Centre for Equid Diseases (CERME). The first case was a 5 years old Italian Heavy Draft horse living semi-feral, never treated against piroplasmosis, the foetus was submitted to determine the cause of the abortion. No sample from the mare were available at the time was available. Due to the breeding system it was difficult to obtain samples so blood samples from the mare were sent 8 months after the abortion, when the animals were moved back from the mountains to lower pastures. It was reported that the mare was never treated and got pregnant with no abnormalities. The second case was an eleven years old Sella Italiano (warmblood breed), a sport horse in active living in a paddock and shelter. Only some foetal remains were found but not the foetus. Mare blood samples, the umbilical cord and placenta were submitted to analyse and another sample of mare blood was sent five months after the abortion. The horse had been treated during pregnancy with imidocarb (10 ml), after the abortion it was treated with tetracycline (dose and drug not reported) and imidocarb (10 ml).

In both cases the abortions were registered in the last trimester. The animals came from two different provinces of Latium (central Italy).

Blood samples were collected from the mares' jugular vein and kept in serum and EDTA tubes. Sera were obtained by centrifugation at 358 g for 10 minutes, then stored at –20°C and thawed at +37°C immediately before testing. Organs (foetal heart, liver, spleen and lung, placenta and umbilical cord) were stored at -80°C until processed.

Foetal heart clot was extracted and stored at –20°C, a part was centrifuged as described before to obtain serum.

To conduct serological tests on the foetal remains from the second abortion case, placenta and umbilical cord were treated to obtain a matrix on which detect antibodies. 1 g. of organ was placed in 2 ml of 1X phosphate buffered saline (PBS) and kept overnight at +4°C, the next day it was hardly vortexed and then centrifuged at 358 g 10 minutes, the supernatant was collected and stored at -20°C (Ferroglio *et al*., 2000).

Microscopic examination

Thin smears from EDTA and contact smears of the organs were prepared and stained with Diff Quick and then observed microscopically (1000x) to determine the presence of intracellular parasites.

Serological tests.

Indirect fluorescent antibody test (IFAT) was used for the detection of specific IgG antibodies against *T. equi* and *B. caballi* infections. Tests were carried out according to the manufacturer's instructions (Fuller Laboratories, Fullerton, California, USA. Samples with a strong fluorescence at a dilution of 1:80 were considered to be positive.

Two commercial competitive enzyme-linked immunosorbent assays (cELISA): *Babesia equi* Antibody test kit VMRD® and *Babesia caballi* Antibody test kit VMRD® were performed according to manufacturers instructions.

Serological tests were conducted on sera and macerated organ supernatant (it was treated and diluted as serum samples were).

PCR amplification protocols and sequencing

a) DNA extraction from blood and organs

DNA extraction from blood was conducted using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) following the protocol described by the manufacturers. The DNA was eluted in 200 µl of buffer AE. DNA yield was determined with a spectrophotometer (Eppendorf BioPhotometer, Eppendorf AG, Hamburg).

To extract DNA from the organs, the same protocol was used adding one previous step: 100 milligrams of organ in 600 µl of 1X PBS were placed in a FastPrep tub and then homogenize in a FastPrep FP120 Cell Disrupter (Thermo Electron) twice at 12000 rpm 5 minutes. Supernatant was collected and use to assess DNA extraction.

b) PCR Protocols used from literature and sequencing

Real time PCR 18S rRNA *B. caballi* amplified a 95bp fragment in the V4 hypervariable region of 18S rRNA gene of *B. caballi*. Primers and probe employed (F: Bc-18SF402; R: Bc-18SR496; Probe: TaqMan MGB™ probe (FAM-MGB), Bc-18SP) were those reported in

literature (Bhoora R. *et al*., 2010). The real rime 18S rRNA *T. equi* amplified an 81bp fragment in the V4 hypervariable region of 18S rRNA gene. Primers and probe employed (F: Be18SF; R: Be18SR) TaqMan probe (VIC-TAMRA, Be 18SP) were those reported in literature (Kim *et al.,* 2008). For both Real Time PCRs, TaqMan® Universal PCR Master Mix kit (A.Biosystems, Foster City, CA, USA) was used. Positive controls were constituted by the plasmid vectors pCRII®-TOPO TA Cloning® Invitrogen, Carlsbad, CA, USA) in which the targets of the real time PCR *T. equi* and *B. caballi* have been cloned.

All real time PCRs were carried out using ABIPRISM 7900 HT Sequence Detection System (A. Biosystems).

Two protocols for *Babesia caballi* called BC48 nested PCR and RAP end point PCR and one protocol for *Theileria equi* called EMA end point PCR were carried out.

The target for BC48 nested PCR was the BC48 gene coding for a 48KDa merozoite protein appointed BC48 and belonging to the complex rhoptry protein. The primers used for the first PCR were BC48F1/BC48R3 (Ikadai *et al.,* 1999a; Battsetseg *et al*., 2002) and amplified a 530 bp fragment of BC48 gene, while the primers for the second PCR were BC48F11/BC48R31 and amplified an internal band of 430 bp (Battsetseg *et al.,* 2001; Battsetseg *et al.,* 2002).

The target for RAP end point PCR was a fragment of 825 bp internal at o rhoptry-associated protein (RAP1) gene and the primers used were BC-RAP2F/ BC-RAP2R (Bhoora *et al.,* 2010).

The target for EMA end point PCR was a 268 bp internal fragment at the merozoite antigen 1 (EMA-1) gene coding for a major parasite surface antigen and the primers used were EMA-5/EMA-6 (Battsetseg *et al.*, 2002).

A nested PCR protocol, that amplifies the hypervariable V4 region of the 18rRNA gene of the genera *Theileria* and *Babesia* was alsp carried out. This protocol has products of approximately 430bp and 390bp for *Theileria* and *Babesia* species respectively, and the primers used are RLB F1/ RLB R1, RLB F2/RLB R2 (Nagore *et al.,* 2004). In order to avoid non-specific reactions, the thermal profile was optimised by changing the annealing temperature from 51°C indicated in literature to 54°C.

AmpliTaq Gold® DNA Polymerase (A. Biosystems, Life Technologies, Austin, Tx, USA) to prepare the master mix and the GeneAmp® PCR System 9700 (A. Biosystems, Foster City, CA, USA) were used In all protocols.

PCR products were visualized after electrophoresis in a 1.5% Tris-Boric Acid-EDTA agarose gel and stained with GelRed 10,000X (Biotium, Hayward, CA, USA).

The PCRs products were recovered from agarose gel using the QIAquick® PCR Purification kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions and sequenced using the PCR primers Bc-18SF402/Bc-18SR496, Be18SF/Be18SR, EMA-5/EMA-6, BC48F11/BC48R31, BC-RAP2F/ BC-RAP2R and RLB F2/RLB R2 with the BigDye Terminator Cycle Sequencing Ready Reaction kit, version 3.1 (PerkinElmer, A. Biosystems, Foster City, CA, USA) in an automated sequencer (3500 Genetic Analyzer, A. Biosystems, Foster City, CA, USA). The nucleotide sequences obtained were analysed using the Genetic Analyzer Sequencing v5.4 (A. Biosystems, Foster City, CA, USA).

Results

Biomolecular results are described in Table 1, serological and microscopic examination results are shown in Table 2.

First case:

No sample of the Italian Heavy Draft mare at the time of the abortion was provided.

Foetus samples (clot, heart, liver, spleen and lung) were positive in PCR with all the protocols detecting *Theilera equi*; positive samples were sequenced and showed 99-100% homology with the accession numbers JQ782603.1 (EMA target) and 91-97% homology with accession numbers JX177673.1 (real time Be18S target). Samples were also positive to the RLB method. The sequences obtained showed 99-100% homology with accession numbers AY150063.1 *(T. equi).* Foetus samples were negative to the BC48 and RAP protocols detecting *B. caballi*, in the real time Bc18S protocol. Clot, heart and lung samples were negative while the spleen was positive. However, no homology was found although when sequenced. The liver sample was positive and the sequence obtained showed a 100% homology with AB734392.2 with a 96% "query coverage".

Blood mare samples and serum arrived to be analysed eight months after the abortion episode, and they tested positive to the real time protocols Bc18S and Be18S, sequences

obtained showed 100% AB734392.2 *(B. caballi)* and AB733379.2 *(T. equi).* They were negative to *B. caballi* RAP protocol and EMA *T. equi* protocol. Faint positivity was observed in the BC48 nested PCR (*B. caballi*) but sequencing was not possible. The sample was positive to the RLB protocol, two bands were detected and sequenced, obtaining a 100% homology to DQ287951.1 (*T. equi*) and 99% AY53882.1 *(B. caballi)*

Blood smears from the mare and the foetus were positive.

Foetal serum was negative to IFAT and ELISA tests. However, serum from the mare was positive to *Babesia caballi* and *Theileria equi* both by IFAT and ELISA tests.

Second case:

Whole blood at the abortion moment from the Sella italiano mare tested positive to real time 18S PCR Bc and Be, sequences obtained showed 99% homology with KJ787774.1 (*B. caballi*) and 100% homology with KJ573373.1 (*T. equi*). It was also positive to the EMA protocol and the sequence showed 100% homology with JQ782603.1. It tested negative for *B.caballi* protocols BC48 and RAP. The sample was positive to the RLB protocol and the sequence showed 99% homology with JX177673.1 *(T.equi).*

Umbilical cord and placenta were positive to real time 18S Be and the sequences obtained showed 95-96% homology with KJ573373.1. Samples were positive to EMA PCR showing 99-100% homology with registered sequences JQ782603.1. Samples tested negative for *B. caballi* protocols BC48 and RAP. Placenta sample was negative in the real time Bc 18S, but umbilical cord tested positive and sequence obtained showed 98% homology with KJ787774.1. In the RLB PCR test samples were positive and sequences obtained showed 86-98% homology with JX679181.1 (*T. equi*).

Table 1: Biomolecular tests results

	RLB PCR		**RAP PCR *B.caballi***	**BC48 PCR *B.caballi***	**REAL TIME *B.caballi***	**EMA5/6 PCR *T.equi***	**REAL TIME *T.equi***
Mare 1 Time 0	NA		NA	NA	NA	NA	NA
Abortion 1 Foetal clot	*T. equi* 100% AY150063		-	-	-	+ 100% JQ782603	+ 97% JX177673
Abortion 1 Foetal heart	*T. equi* 99% AY150063		-	-	-	+ 99% JQ782603	+ 97% JX177673
Abortion 1 Foetal liver	*T. equi* 99% AY150063		-	-	+ 100% AB734392	+ 99% JQ782603	+ 93% JX177673
Abortion 1 Foetal spleen	*T. equi* 99% AY150063		-	-	+ No homology	+ 99% JQ782603	+ 91% JX177673
Abortion 1 Foetal lung	*T. equi* 99% AY150063		-	-	-	+ 99% JQ782603	+ 91% JX177673
Mare 1 Time 2	*T. equi* 100% DQ287951	*B.caballi* 99% AY534883	-	Faint band	+ 100% AB734392	-	+ 100% AB733379
Mare 2 Time 0	*T. equi* 99% JX177673		-	-	**+** 100% KJ787774	**+** 100% JQ782603	**+** 100% KJ573373
Abortion 2 Umbilical cord	*T. equi* 86% JX679181		-	-	+ 98% KJ787774	**+** 99% JQ782603	**+** 95% KJ573373
Abortion 2 Placenta	*T. equi* 98% JX679181		-	-	-	**+** 100% JQ782603	**+** 96% KJ573373
Mare 2 Time 2	*T. equi* 99% KM046921	*B.caballi* 98% EU888904	ND	ND	+ 100% KJ787774	-	**+** 100% KJ573373

ND: not done; NA sample not available; + positive result; - negative result;

A second blood sample provided five months after resulted negative to EMA PCR protocol, but positive to real time 18S Be, sequence obtained showed 100% homology with KJ573373.1. The Bc real time tested positive and sequence was 100% KJ787774.1. RAP and BC48 protocols were not performed. In the RLB protocol two bands were observed, sequences showed 99% homology to registered sequences KM046921.1 (*T.equi)* and 98% homology to registered sequences EU888904.1 *(B.caballi).*

Foetal remains and mare blood smears examined microscopically were positive. The macerate tissue supernatants were negative to all serological tests. No serum sample from the mare at the moment of abortion was provided, but a serum sample sent five months after the abortion tested positive to *B.caballi* and *T.equi* by IFAT but negative to both by ELISA.

Table 2: Smears and serological results

	Smear	**IFAT *B.caballi***	**IFAT *T.equi***	**ELISA *B.caballi***	**ELISA *T.equi***
Mare 1. Time 0	NA	NA	NA	NA	NA
Abortion 1. Foetal clot/serum	+	-	-	-	-
Mare 1. Time 2	+	+	+	+	+
Mare 2. Time 0	+	NA	NA	NA	NA
Abortion 2. Umbilical cord	+	-	-	-	-
Abortion 2 Placenta	+	-	-	-	-
Mare 2. Time 2	+	+	+	-	-

NA sample not available; + positive result; - negative result;

Discussion

Both Italian bred mares came from different enviroments and developed different aptitudes. Being field samples and in particular breeding conditions it was difficult to follow up the cases and perform more sampling.

In the first case a foetus was examined and different PCR protocols were conducted on

the organs and clot. *T.equi* protocols were positive and the sequences obtained were similar among different tissues. Comparable results were obtained using RLB protocol. Real time *B. caballi* method was positive in the liver and spleen samples but homology with *B. caballi* was only found just in the liver sequenced product. The end point *B.caballi* protocols were negative. Blood stains were positive and serological tests were all negative.

No sample from the Heavy Draft mare was collected at the time of the abortion, but one was sent 8 months after. It was positive to the real time protocols showing the same sequence as the foetus for *B. caballi.* RLB protocol was positive and two products were sequenced, one was *B. caballi* and the other *T. equi* but the sequences were slightly different from the foetus one. EMA PCR was negative, also RAP PCR, however a faint and was observed in the nested *B. caballi* protocol. Blood stains and serological tests were positive.

In the second case, the foetus was not available for examination but foetal remains were found. The blood of the Sella italiano mare was provided at the time of the abortion. Blood stains and contact stains evidenced the presence of piroplasms. *Theileria equi* was detected using different target PCR protocols, the accession numbers correspondent to the product sequencing were similar among the foetal cord, placenta and mare blood. However, sequences obtained with the RLB amplification protocol were different, the same strain was evidenced in the foetal cord and placenta but it was different about 4% from the mare strain. *Babesia caballi* end point protocols could not detect the parasite although *B. caballi* real time protocol was positive in the cord and in the mare and the same sequence accession number was observed.

A second sample of the Sella Italiano mare was sent for analysis 5 months after the abortion and it resulted positive to the real time protocols. Products were sequenced and sequences obtained were similar to those observed at the time of the abortion in mare and foetal remains. With the RLB amplification method but it was possible to individuate two bands which were sequenced as *B. caballi* and *T. equi.* The *T.equi* sequence was different from those found in the cordon and from the first sequence of the mare.

Babesia caballi end point protocols could not detect the parasite. It may be due to the low parasitemia and the fact that amplicons are very long (400-800 aprox.). The EMA protocol was negative.

Due to the lack of foetal serum, the protocol described above was used to obtain

antibodies, no sample was positive to the serological methods, but the serum of the second draw blood was positive to both piroplasms using IFAT. Surprisingly, all samples were negative by ELISA, which should be further investigated.

Some results are common in both cases. The negativity to EMA PCR in the second sample could be related to a lack of expression of the EMA gene. Some related conducted by Kumar *et al.* (2004) showed differences in expression in life cycle of the parasite.

Differences observed among sequences from first and second samplings in both abortion cases could be explained because RLB protocol catches several forms of *T. equi* and *B. caballi*, we can evidence it by sequencing. However, when several variants coexist, the sequence obtained is the predominant form so if more strains are present could be masked by the predominant one. It may occur that it is not even possible to distinguish among species when in the same band. In our abortion cases it could be possible that these variant forms were all present but more predominant in different tissues or moments of the parasite life cycle. Another explanation could be a reinfection with different piroplasms forms.

The lack of antibodies in the infected foetuses and its acquisition with the colostrum have been described by Kumar *et al*. (2008).

This is the first evidence of transplacental transmission of *B. caballi* in Europe, it was only possible using real time protocols, *Babesia caballi* end point protocols couldn't detect the parasite may be due to the low parasitemia and the fact that amplicons are very long (400-800 aprox.), remaining under the detection limit agreeing to the observations reported by Sant *et al.* (2016). Real time amplicons are much shorter which overcomes the problem of DNA degradation. The tissues, where the positive results were found, have physiological specific characteristics and are more perfused than the others so more parasites could be found.

Conclusion

This study is the first evidence of transplacental transmission of both piroplasms *B.caballi* and *T.equi.*

Piroplasms vertical transmission is an underestimated problem, it causes abortion and neonatal mortality generating important economic losses. Appropriate preventive measures should be advised to manage infected pregnant mares.

Further investigation about transplacental transmission mechanisms in endemic areas, the role to maintain infections and new strategies of treatment for carriers are needed to overcome this problem.

Acknowledgements

This study was developed with funds from the IZSLT 16/11 project (provided by the Italian Ministry of Health).

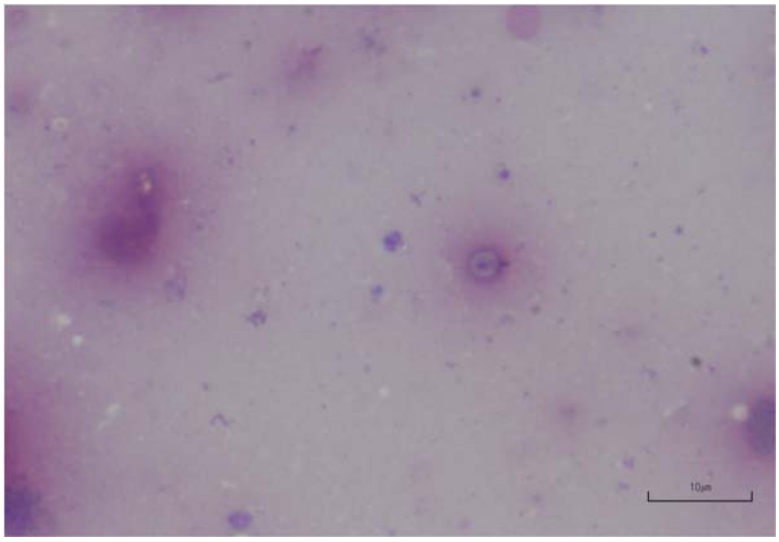
Conflict of interest

The author declare no conflict of interests.

Author contribution

Development of assays, DNA extraction and purification, sequences alignment and manuscript redaction.

Image: Blood smear from foetus heart clot



References

Allsopp MTEP, Lewis BD, Penzhorn BL: Molecular evidence for transplacental transmission of *Theileria equi* from carrier mares to their apparently healthy foals. *Vet Parasitol* **2007**, 148:130–136.

Battsetseg B, Lucero S, Xuan X, Claveria FG, Inoue N, Alhassan A, Kanno T, Igarashi I, Nagasawa H,

Mikami T, Fujisaki K: Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. *Vet Parasitol* **2002**, 107:351–357.

Bhoora R, Quan M, Franssen L, Butler CM, Van der Kolk JH, Guthrie AJ, Zweygarth E, Jongejan F, Collins NE: Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. *Vet Parasitol* **2010**, 168:201–211.

Bhoora R, Quan M, Zweygarth E, Guthrie AJ, Prinsloo S, Collins NE: Sequence heterogeneity in the gene encoding the rhoptry-associated protein-1 (RAP-1) of *Babesia caballi* isolates from South Africa. *Vet Parasitol* **2010**, 169:279–288.

Chhabra S, Ranjan R, Uppal SK, Singla LD: Transplacental transmission of *Babesia equi (Theileria equi*) from carrier mares to foals. *J Parasit Dis* **2012**, 36:31–33.

Ferroglio E, Rossi L, Gennero S: Lung-tissue extract as an alternative to serum for surveillance for brucellosis in chamois. *Prev Vet Med* **2000,** 43:117–122.

Georges KC, Ezeokoli CD, Sparagano O, Pargass I, Campbell M, D'Abadie R, Yabsley MJ: A case of transplacental transmission of *Theileria equi* in a foal in Trinidad. *Vet Parasitol* **2011**, 175:363–366.

Ikadai H, Xuan X, Igarashi I, Tanaka S, Kanemaru T, Nagasawa H, Fujisaki K, Suzuki N, Mikami T: Cloning and expression of a 48-kilodalton *Babesia caballi* merozoite rhoptry protein and potential use of the recombinant antigen in an enzyme- linked immunosorbent assay. *J Clin Microbiol* **1999**, 37:3475–3480.

Kim CM, Blanco LBC, Alhassan A, Iseki H, Yokoyama N, Xuan X, Igarashi I: Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. *Vet Parasitol* **2008**, 151:158–163.

Kumar S, Yokoyama N, Kim JY, Huang X, Inoue N, Xuan X, Igarashi I, Sugimoto C: Expression of *Babesia equi* EMA-1 and EMA-2 during merozoite developmental stages in erythrocyte and their interaction with erythrocytic membrane skeleton. *Mol Biochem Parasitol* **2004**, 133:221–227.

Kumar S, Kumar R, Gupta a. K, Dwivedi SK: Passive transfer of *Theileria equi* antibodies to neonate foals of immune tolerant mares. *Vet Parasitol* **2008,** 151:80–85.

Nagore D, García-Sanmartín J, García-Pérez AL, Juste R a., Hurtado A: Detection and identification of equine *Theileria and Babesia* species by reverse line blotting: Epidemiological survey and phylogenetic analysis. *Vet Parasitol* **2004,** 123:41–54.

Sant, C., d'Abadie, R., Pargass, I., Basu, A.K., Asgarali, Z., Charles, R.A., Georges, K.C., Prospective study investigating transplacental transmission of equine piroplasmosis in thoroughbred foals in Trinidad. *Vet. Parasitol*. **2016**. 226, 132–137.

CAPÍTULO 3: PARÁMETROS HEMÁTICOS Y BIOQUÍMICOS EN LA PIROPLASMOSIS EQUINA

CHAPTER 3: HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN EQUINE PIROPLASMOSIS

CAPITOLO 3: PARAMETRI EMATICI E BIOCHIMICI NELLA PIROPLASMOSI EQUINA

RESUMEN CAPÍTULO 3

En este tercer capítulo se recogen datos sobre los valores hemáticos y bioquímicos de dos grupos de animales con una sintomatología compatible con piroplasmosis, el primer grupo está constituido por muestras de caballos analizadas en el Hospital Clínico Veterinario de la UCM y el segundo por muestras de équidos analizadas en el IZSLT. Se determinó una batería de parámetros hemáticos y bioquímicos y se hicieron test de IFI en ambos grupos y de frotis sanguíneo (solo en IZSLT) para diagnosticar la enfermedad. Mediante el test de Wlicoxon se observó la presencia de diferencias estadísticamente significativas entre los valores obtenidos en animales positivos y negativos a piroplasmosis, con el objetivo de evidenciar un parámetro útil como biomarcador de piroplasmosis equina ya que los signos clínicos y alteraciones sanguíneas que presenta esta enfermedad son muy inespecíficos.

Se determinaron alteraciones hematológicas en la serie roja, blanca así como en plaquetas; los parámetros bioquímicos también mostraron variaciones; se determinaron diferencias significativas entre animales positivos y negativos, pero no se ha podido establecer ningún marcador biológico ya que estas diferencias significativas no se presentaban en todas las técnicas diagnósticas o estaaban en parámetros dentro del rango.

Contribución de la autora: recolección y organización de datos sobre los valores hemato-bioquímicos y diagnósticos y redacción del texto.

RIASSUNTO CAPITOLO 3

In questo terzo capitolo sono stati raccolti dati sui parametri ematici e biochimici di due gruppi di animali con sintomatologia compatibile con piroplasmosi; il primo gruppo è costituito da campioni di cavalli analizzati nel'Ospedale Clinico Veterinario della UCM e il secondo da campioni di equini analizzati nell' IZSLT.

È stata determinata una batteria di parametri ematici e biochimici e sono stati fatti test di IFI in ambi i gruppi e strisci sanguigni (solo nell'IZSLT) per diagnosticare la malattia. Utilizzando il test di Wilcoxon si osservò la presenza di differenze statisticamente significative fra i valori ottenuti negli animali positivi e negativi a piroplasmosi, con l'obiettivo di evidenziare un parametro utile come biomarcatore della piroplasmosi equina poiché i segni clinici e le alterazioni sanguigne che presenta questa malattia non sono molto specifici.

Sono state determinate alterazioni ematologiche nella serie rossa e bianca così come nelle piastrine; i parametri biochimici inoltre mostrano diverse variazioni; sono state determinate differenze significative fra animali positivi e negativi, ma non si è potuto stabilire nessun biomarcatore perché le alterazioni non si evidenziavano in tutte le tecniche diagnostiche o si presentavano in parametri dentro il range.

Contributo dell'autrice: ricollocazione e organizzazione dei risultati sui dati emato-biochimici e diagnostici. Realizzazione delle PCR e IFI, estrazzione e purificazione del DNA, analisi delle sequenze e redazione del testo.

STUDY 3

Haematological and biochemical parameters in animals infected with equine piroplasmosis

Abstract

The first group consisted of samples (n=23) of horses analysed in the Clinical Veterinary Hospital of the UCM and the second consisted of samples of equidae analysed in the IZSLT (n=101). A set of haematological and biochemical parameters were determined and IFAT tests and blood smears (at IZSLT only) were performed in both groups to diagnose the disease. Haematological alterations were observed in the red and white series as well as in platelets; biochemical parameters also showed different variations. The Wilcoxon test determined the presence of statistically significant differences among the values obtained in positive and negative animals, but no biological marker could be established since these significant differences were not present in all the diagnostic techniques or were evidenced in parameters within the range.

Introduction

Babesioses clinical symptoms are nonspecific and resemble those of other diseases so diagnostic tests must be performed to rule out other disorders. The aim of this study was to evaluate the differences among haemato-biochemical parameters in symptomatic animals tested positive and negative to equine piroplasmosis and the possibility to use some of these parameters as specific biomarkers.

Most frequent alterations of red cells are: low erythrocyte count, hemoglobin and hematocrit, and relative variations in RDW, MCV, MCH and MCHC that occur in the course of microcytic and hypochromic anemia. The activity of the hematopoietic organs can also be altered by releasing poikilocytes and macroeritrocytes (Mahmoud *et al*., 2016; Sumbria *et al*., 2017). As for the white cells, the authors' observations have been variable finding cases of leukopenia and leukocytosis, neutropenia and neutrophilia, lymphopenia or lymphocytosis (Al-Saad *et al*., 2009: Laus *et al*., 2015) and monocytosis (Diana *et al.* 2007). Platelet count is low, resulting from local and systemic disseminated intravascular coagulation, immune-

mediated destruction and blocking of platelets in the spleen (Beard *et al*., 2013). Other alterations in coagulation parameters such as plasma fibrinogen, D-dimer, coagulation time, prothrombin time, activated partial thromboplastin have been described by different authors (Mantran *et al.,* 2004, A-Saad *et al*., 2009).

Other frequent findings are increased bilirubin caused by hemolysis (Al-Saad *et al*., 2009), elevated hepatic transaminases due to central-lobular degeneration and hepatocyte necrosis (Camacho *et al*., 2005; Zobba *et al.,* 2008), and hypoproteinemia with hypoalbuminemia and alterations in α2- and γ- globulins (Rubino *et al*., 2006; Barrera *et al*., 2010).

Materials and methods

The study was conducted on two groups of symptomatic equids, one in Spain (n=23) and one in Italy (n=101). Whole blood and EDTA samples were collected from the animals and sera were obtained by centrifugation at 358 g for 10 minutes.

In the Italian group red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell volume distribution width (RDW), platelet (PLT), mean platelet volume (MPV), plateletcrit (PCT), plate volume distribution width (PDW), white blood cells (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO), T-helper lymphocytes (CD4+), cytotoxic T lymphocytes (CD8+) and ratio of T-helper cells to cytotoxic T cells (CD4+/CD8+) on EDTA blood were determined using an automated Cell-Dyn 3700® (Abbott). Sera aspartate aminotransferase (AST), blood urea nitrogen (BUN), direct bilirubin (DIRECT BIL), total bilirubin (TOT BIL), creatine kinase (CK), creatine (CREA), gamma glutamyltransferase (GGT), lactate dehydrogenase (LDH) and total proteins (TOTAL PROT) were measured by an automatic biochemical analyser (Olympus AU 400); indirect bilirubin (INDIRECT BIL) was calculated as the difference between total and direct bilirubin. Albumin (ALB), alpha 1 globulins (ALPHA 1), alpha 2 globulins (ALPHA 2), beta globulins (BETA), gamma globulins (GAMMA) and ratio albumin/globulins (A/G) were determined by serum protein electrophoresis using a P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Inc., Fullerton, California, USA). Total antioxidants and free radicals were monitored by a colorimetric method (DIACRON).

In the Spanish group EDTA samples were submitted for a complete blood count, which

included: hematocrit (HCT), hemoglobin (HGB), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT), white blood cells (WBC), neutrophils (NEU), band neutrophils (BAND NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS) and basophils (BASO).

Blood urea nitrogen (BUN), creatine (CREA), total proteins (TOTAL PROT), indirect bilirubin (INDIRECT BIL), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT) and glutamate-pyruvate transaminase (GPT) were measured on sera.

Indirect fluorescent antibody test (IFAT) was used for the detection of specific IgG antibodies against *T. equi* and *B. caballi* infections. Tests were carried out according to the manufacturer's instructions (Fuller Laboratories, Fullerton, California, USA). Samples with a strong fluorescence at a dilution of 1:80 were considered to be positive.

In the Italian group piroplasms were also diagnosed microscopically in Giemsa-stained films of EDTA-anticoagulated blood. All fields were examined at x1000 magnification before the sample was declared free of piroplasms.

Wilcoxon statistical test was used to determine the presence of statistically significant differences among values present in positive and negative animals. A p value <0.05 was considered statistically significant. SAS 9.4 software for Windows was used for all statistical analyses.

Results

Spanish group

Haematological parameters in positive animals to *B.caballi* IFAT showed a decrease in RBC, HB, HTC and LYM and an increase in MCHC, WB and NEU. Regarding biochemistry parameters only INDIRECT BIL resulted higher for *B.caballi* positive animals. No statistical differences between these parameters and those from negative animals were found. MONO values resulted statistically different between positive and negative horses being higher in the negative ones although mean values were within the range.

For *T. equi* positive animals, transaminase values showed alterations, observing lower AST and higher GPT levels. The INDIRECT BIL parameter was higher than in negative animals.

No statistically significant differences were observed between positive and negative animals (Table 1).

Italian group

In positive animals for *B.caballi* IFAT, *T.equi* IFAT and blood smear HB, HTC and PLT values showed a decrease while RDW, TOT BIL, INDIRECT BIL, LDH free radicals and antioxidants levels were increased. Protein electrophoresis showed low alpha1 globulins and high gamma globulins levels.

Decreased RBC and increased WB, EOS parameters in *T. equi* IFAT and blood smear positive equids were found.

Significant differences were observed between several altered mean values from infected animals diagnosed by blood smear or *T. equi* IFAT and negative animals; PDW, LYM, MONO, BASO, CK, ALB and ratio ALB/GLOB also showed significant differences too but values were within the range.

No statistically differences for *B. caballi* IFAT positive and negative animals were observed except for PDW still values fit in the normal range (more information in Table 2).

Discussion and conclusions

Equine piroplasmosis is a febrile-hemolytic syndrome; three mechanisms of hemolysis have been described: mechanical by trophozoite intra-erythrocyte binary fission, immune-mediated by auto-antibodies directed against components of the membranes of infected and uninfected erythrocytes, and toxicity by hemolytic factors produced by the parasite (Wise *et al.*, 2014). Due to hemolysis HTC, RDW, MCV, MCH and MCHC parameters show decreased levels, as previously described by different authors (Mahmoud *et al.*, 2016; Adam *et al.*, 2017; Sumbria *et al*., 2017)

In this study low PLT levels were observed; thrombocytopenia is a common finding in EP (Al-Saad *et al.,* 2009; Laus *et al.,* 2015), it may be caused by local and systemic disseminated intravascular coagulation, immune-mediated destruction, and sequestration of platelets in the spleen (Beard *et al.*, 2013).

White cells parameters could show different alterations such as leukocytosis-leukopenia, neutropenia and neutrophilia, lynphopenia and lynfocytosis (Al-Saad *et al.,* 2009; Laus *et al.,* 2015) and monocytosis (Diana *et al.,* 2007).

Other common findings, also evidenced in our results are increased bilirubin caused by hemolysis (Al-Saad *et al.,* 2009): high liver enzymes associated with necrosis of hepatocytes and centrilobular degeneration (Camacho *et al.,* 2005; Zobba *et al.,* 2008) and alterations in α2 globulins and γ globulins (Rubino *et al.,* 2006; Barrera *et al.,* 2010).

In our study antioxidants and free radicals presented high levels due to red blood cells damage, this finding agress with other authors who have reported altered values of oxidative stress and lipid peroxidation markers (Ambawat *et al.*, 1999; Deger *et al.*, 2009).

The haemato-biochemical changes observed were similar in both groups although clinical forms in the Italian group were chronic and acute and in the Spanish group were predominantly chronic. In the Spanish group few alterations (BIL and liver enzymes) and statistically significant differences among positive *T. equi* IFAT and negative animals were found. These findings may suggest that maybe these animals were recently seropositive and not carriers as *T. equi* antibodies are long lasting (Wise *et al*. 2014). The lack of *B. caballi* significant results could be due to the low number of positive samples.

Due to the unspecific clinical signs that appear in EP affected horses, the aim of this study was to evaluate haemato-biochemicals alterations in infected animals and its differences with non infected ones. No biological marker could be established since statistically significant differences were not present in all the diagnostic techniques or were evidenced in parameters within the range.

Acknowledgements

Part of this study was developed with funds from the IZSLT 16/11 project (provided by the Italian Ministry of Health).

Conflict of interests

The author declares no conflict of interests.

Author contribution

Data selection, cleaning and organization, partial analyse and manuscript redaction.

Table 1: Haemato-biochemicals parameters in the Spanish group. (*alterated parameter; bold characters show significant parameters)

	IFAT BABESIA CABALLI				IFAT THEILERIA EQUI			
HAEMATO-BIOCHEMICAL PARAMETERS AND RANGE	NEG (n=20)		POS (n=3)		NEG (n=14)		POS (n=9)	
	Mean	Std dev	Mean	Std dev	Mean	Std dev	Mean	Std dev
HCT (32-53%)	34,18	10,28	27,43*	5,86	32,10	5,79	35,17	14,32
HGB (11-19 g/dl)	11,53	3,44	9,3*	1,13	10,84*	2,34	12,09	4,62
RBC (6,8-12,9x10^6/µl)	7,19	1,75	5,67*	0,67	7,02	1,63	7,10	1,99
MCV (37-58,5 fl)	48,37	7,14	48,10	5,94	47,30	5,12	50,04	9,26
MCH (12,3-19,7 pg)	16,06	2,35	16,35	0,07	15,62	2,04	16,84	2,45
MCHC (31-37 g/dl)	36,03	11,58	37,4*	0,00	37,45*	13,84	34,06	2,97
PLT (100-350x10^3/µl)	166,55	59,24	229,50	120,92	176,00	65,98	176,60	80,55
WBC (5,4-14,3x10^3/µl)	7,82	2,92	15,27*	9,70	8,41	3,06	9,38	6,79
NEU (2260-8580 /µl)	3675,90	2562,40	14163*	9459,90	4028,70	2787,00	6622,80	7523,30
BAND NEU (0-100 /µl)	40,20	114,83	0,00	2,48	55,79	135,52	2,56	7,67
LYM (1500-7700 /µl)	2728,80	1384,30	1371,7*	342,03	2873,30	1591,10	2051,60	777,44
MONO (0-1000 /µl)	**246,40**	**234,64**	**0,00**	**0,00**	272,43	270,53	123,78	128,45
EOS (0-1000 /µl)	92,95	112,70	0,00	0,00	97,07	124,61	55,56	81,07
BASO (0-290 /µl)	6,00	26,83	0,00	0,00	8,57	32,07	0,00	0,00
BUN (26-55 mg/dl)	34,77	9,64	36,50	7,78	33,56	8,81	37,17	10,13
CREA (1-1,9 mg/dl)	1,11	0,73	1,60	0,14	1,10	0,93	1,37	0,31
TOTAL PROT (5,5-7,3 g/dl)	6,51	0,97	7,00	0,87	6,47	1,14	6,73	0,59
INDIRECT BIL (0,5-2,1 mg/dl)	3,51*	2,31	3,6*	0,00	3,92*	2,48	2,73*	1,41
AST (152-296 U/L)	119,59	67,27	208,00	0,00	129,32*	76,39	129,6*	68,72
GGT (9-25 U/L)	11,31	9,69	4,00	0,00	12,18	10,57	5,49	2,10
GPT (4-12 U/L)	172,67*	205,07	356,25*	23,60	55,5*	41,72	407*	0,00

Table 2: Haemato-biochemicals parameters in the Italian group. (*alterated parameter; bold characters show significant parameters)

	IFAT BABESIA CABALLI				IFAT THEILERIA EQUI				BLOOD SMEAR			
HAEMATO-BIOCHEMICAL PARAMETERS AND RANGE	NEG (n=92)		POS (n=9)		NEG (n=59)		POS (n=42)		NEG (n=78)		POS (n=18)	
	Mean	Std dev	Mean	Std dev	Mean	Std dev	Mean	Std dev	Mean	Std dev	Mean	Std dev
ANTIOXIDANTS (200- 300 µg/dl)	399,78*	154,33	402,63*	243,48	**295,57**	**141,41**	**443,73***	**170,23**	433,56*	152,66	468,73*	172,44
AST (200-400 U/L)	305,48	151,02	373,50	160,82	299,57	47,69	327,32	166,89	371,90	282,53	293,28	142,97
BUN (10-25 mg/dl)	13,14	2,63	16,75	10,35	13,14	1,95	14,36	6,44	14,83	3,57	14,61	7,29
DIRECT BIL (0-0,4 mg/dl)	0,50*	0,30	1,24*	1,77	0,55*	0,15	0,75*	1,05	0,52*	0,23	0,74*	1,24
TOT BIL (0,2-6 mg/dl)	2,12	1,42	5,00	4,55	2,46	1,86	3,42	3,40	2,05	1,40	3,45*	4,02
INDIRECT BIL (0,5-2,5 mg/dl)	1,62	1,16	3,75*	2,94	1,92	1,72	2,66*	2,60	1,58	1,23	2,71*	3,07
CK (0-500 U/L)	260,48	145,13	333,63	308,06	**390,14**	**170,13**	**234,48**	**189,33**	320,41	443,29	252,83	228,01
CREA (0,6-1,8 mg/dl)	1,18	0,23	1,24	0,33	1,32	0,23	1,16	0,24	1,32	0,31	1,22	0,28
GGT (0-25 U/L)	41,8*	66,89	15,00	4,90	14,29	6,87	37,04	61,75	20,81	34,81	23,59	18,98
LDH (0-350 U/L)	551,1*	228,22	826,5*	567,09	552,14*	257,95	696,12*	522,02	512,74*	241,27	741,78*	592,63
FREE RADICALS (44-88 U.C)	212,24*	114,41	270,00*	153,83	163,86*	95,11	238,52*	125,58	**154,08***	**89,76**	**245,94***	**133,51**
CD4+ (0-56 n°/µl)	40,04	5,38	40,62	7,86	40,99	4,82	39,68	6,26	38,09	9,59	41,29	6,50
CD8+ (20-37 n°/µl)	16,61	4,62	16,67	6,67	19,44*	4,46	15,95*	5,19	16,5*	5,07	17,23	5,57
CD4+/CD8+ (0-4,75)	2,65	1,13	3,13	2,18	2,19	0,52	2,89	1,53	2,53	1,19	2,77	1,46
WBC (6-10x10^3/µl)	10,54*	5,36	8,71	3,16	7,78	1,02	10,63*	5,02	**8,46**	**3,08**	**10,10***	**3,81**
NEU (2,7-7,6x10^3/µl)	5,72	5,19	5,10	2,90	4,47	0,96	5,93	4,89	5,59	2,70	4,92	2,72
LYM (1,5-4,5x10^3/µl)	3,03	2,62	2,25	1,12	2,23	0,58	2,93	2,41	**1,98**	**0,96**	**3,40**	**2,74**
MONO (0-0,8x10^3/µl)	1,15*	1,13	1,19*	1,19	0,86*	0,91	1,23*	1,16	**0,61**	**0,72**	**1,17**	**1,06**
EOS (0-0,4x10^3/µl)	0,54*	0,81	0,13	0,10	0,20	0,08	0,45*	0,75	0,30	0,31	0,52*	0,89
BASO (0-0,1x10^3/µl)	0,10	0,15	0,06	0,04	0,04	0,02	0,10	0,14	**0,04**	**0,05**	**0,09**	**0,14**
RBC (6-9,9x10^6/µl)	6,45	1,78	6,47	1,40	**7,85**	**0,81**	**5,92***	**1,65**	**7,05**	**1,37**	**5,54***	**1,49**
HGB (11-15 g/dl)	11,47	2,66	10,89*	2,45	**13,42**	**1,57**	**10,46***	**2,54**	**12,25**	**1,97**	**9,92***	**2,27**
HCT (33-42%)	33,93	8,04	32,15*	7,68	**39,28**	**4,72**	**31,01***	**7,96**	**36,15**	**5,83**	**29,54***	**7,34**
MCV (37-59 fl)	53,53	7,04	49,63	4,70	50,08	2,57	53,01	6,87	51,79	4,07	54,13	8,15
MCH (13-20 pg)	18,09	2,03	16,79	0,92	17,08	0,74	17,92	1,95	17,55	1,29	18,22	2,33
MCHC (31,3-39 g/dl)	33,88	0,80	33,93	1,80	34,13	0,70	33,89	1,20	33,93	0,75	33,76	1,40
RDW (0-20%CV)	23,48*	1,47	23,69*	1,26	23,90	1,46	23,46*	1,34	24,10*	1,63	23,49*	1,47
PLT (100-350x10^3/µl)	115,52	68,67	73,34*	55,21	129,22	47,96	94,17*	68,67	**134,69**	**42,37**	**84,12***	**66,55**
MPV (0-99,9 fl)	8,75	1,82	11,28	2,16	8,06	0,93	9,42	2,34	**8,08**	**1,77**	**10,30**	**3,51**
PCT (0-9,99 %)	0,16	0,13	0,11	0,07	0,12	0,02	0,14	0,12	0,12	0,07	0,11	0,06
PDW (0-99,9 10-GSD)	**18,79**	**1,50**	**21,40**	**1,39**	18,40	0,77	19,49	2,00	**18,44**	**1,49**	**20,08**	**2,30**
TOTAL PROT (5,2-7,9 g/dl)	6,66	0,64	6,70	0,71	6,31	0,17	6,77	0,69	6,49	0,71	6,80	0,71
ALB (2,6-3,7 g/dl)	2,87	0,27	2,90	0,35	**3,06**	**0,22**	**2,81**	**0,27**	2,85	0,43	2,73	0,37
ALPHA 1 (0,2-0,4 g/dl)	0,10*	0,02	0,10*	0,02	0,10*	0,02	0,10*	0,02	0,10*	0,02	0,10*	0,02
ALPHA 2 (0,5-1 g/dl)	0,89	0,13	0,87	0,13	0,88	0,08	0,86	0,15	0,85	0,16	0,88	0,16
BETA (0,7-2,4 g/dl)	0,70	0,12	0,79	0,15	0,70	0,09	0,73	0,14	0,80	0,39	0,72	0,12
GAMMA (0,5-1,9 g/dl)	2,1*	0,77	2,05*	0,47	**1,58**	**0,15**	**2,27***	**0,71**	**1,89**	**0,50**	**2,37***	**0,73**
A/G (0,64-1,36)	0,79	0,18	0,77	0,12	**0,94**	**0,10**	**0,73**	**0,15**	**0,82**	**0,23**	**0,69**	**0,16**

References

Adam, M., Pikalo, J., Snyder, A., Steinrigl, A., Köller, G., Schusser GF. Equine Piroplasmosis – a case of severe *Babesia caballi* infection associated with acute renal failure. **2017**. 4, 113–118.

Alsaad, K.M., Alsaad, E.A., Al-Derawie, H.A. Clinical and Diagnostic Study of Equine Babesiosis in Drought Horses in Some Areas of Basrah Province. *Research Journal of Animal Sciences*, **2010.** 4: 16-22.

Ambawat, H.K., Malhotra, D. V., Kumar, S., Dhar, S. Erythrocyte associated haemato-biochemical changes in *Babesia equi* infection experimentally produced in donkeys. *Vet. Parasitol.* **1999**.85, 319–324.

Barrera, R.; Carapeto, M. V.; Habela, M. A.; Zaragoza, C. Electrophoretic pattern of serum proteins in horses with babesiosis. *Arch. Med. Vet.* **2010**, *42*, 173–178.3.

Beard, L. a, Pelzel, A.M., Rush, B.R., Wright, A.M., Galgut, B.I., Hennager, S.G., King, A.O., Traub-Dargatz, J.L. *Babesia equi*-induced anemia in a Quarter Horse and subsequent regulatory response*. J. Am. Vet. Med. Assoc*. **2013**. 242, 992–6.

Camacho, A.T., Guitian, F.J., Pallas, E., Gestal, J.J., Olmeda, A.S., Habela, M.A., Telford, S.R., Spielman, A., 2005*. Theileria (Babesia) equi* and *Babesia caballi* infections in horses in Galicia, Spain. *Trop. Anim. Health Prod*. 37, 293–302.

de Waal, D.T.. Equine piroplasmosis: a review*. Br. Vet. J.* **1992**. 148, 6–14.

Deger, S., Deger, Y., Bicek, K., Ozdal, N., Gul, A. Status of Lipid Peroxidation, Antioxidants, and Oxidation Products of Nitric Oxide in Equine Babesiosis: Status of Antioxidant and Oxidant in Equine Babesiosis*. J. Equine Vet. Sci.* **2009**. 29, 743–747.

Diana, A., Guglielmini, C., Candini, D., Pietra, M., Cipone, M. Cardiac arrhythmias associated with piroplasmosis in the horse: A case report*. Vet. J*. **2007**. 174, 193–195.

Laus, F., Spaterna, A., Faillace, V., Veronesi, F., Ravagnan, S., Beribé, F., Cerquetella, M., Meligrana, M., Tesei, B. Clinical investigation on *Theileria equi* and *Babesia caballi* infections in Italian donkeys*. BMC Vet. Res*. **2015**. 11, 100.

Mahmoud, M.S., El-Ezz, N.T.A., Abdel-Shafy, S., Nassar, S.A., El Namaky, A.H., Khalil, W.K.B., Knowles, D., Kappmeyer, L., Silva, M.G., Suarez, C.E. Assessment of *Theileria equi* and *Babesia caballi* infections in equine populations in Egypt by molecular, serological and

hematological approaches. *Parasit. Vectors***. 2016***.* 9, 260.

Rubino, G., Cito, A.M., Lacinio, R., Bramante, G., Caroli, A., Pieragostini, E., Petazzi, F. Hematology and some blood chemical parameters as a function of tick-borne disease (TBD) signs in horses*. J. Equine Vet. Sci.* **2006**. 26, 475–480.

Sumbria, D., Singla, L.D., Sharma, A., Bal, M.S., Randhawa, C.S. Veterinary Parasitology : Regional Studies and Reports Molecular survey in relation to risk factors and haemato-biochemical alteration in *Theileria equi* infection of equines in Punjab Province, India. *Vet. Parasitol. Reg. Stud. Reports.* **2017**. 8, 43–50.

Wise, L. N.; Pelzel-McCluskey, A. M.; Knowles, D. P. Equine Piroplasmosis. *Vet. Clin. North Am. Equine Pract.* **2014**, *30*, 677–693.

Zobba, R., Ardu, M., Niccolini, S., Chessa, B., Manna, L., Cocco, R., Pinna Parpaglia, M.L. Clinical and Laboratory Findings in Equine Piroplasmosis. *J. Equine Vet. Sci*. **2008**. 8, 301–308.

EPILOGO

El presente epílogo recoge las discusiones presentadas en cada estudio.

La ausencia de una prueba única para el diagnóstico fiable de la piroplasmosis y el hecho que las técnicas de diagnóstico aconsejadas por la OIE sean solo de tipo serológico hace difícil la elección de un método especialmente cuando necesitamos discriminar entre animales solo serológicamente positivos, portadores sanos o animales con formas clínicas agudas en los que aún no se han producido anticuerpos específicos. En áreas donde la enfermedad es endémica es imprescindible diferencias entre la serodetección, la detección de parásitos (o su ADN) en animales portadores y el diagnóstico de un animal enfermo. En estos casos, el uso único de técnicas serológicas no nos permite diferenciar animales expuestos de portadores y enfermos y necesitamos complementarlas para saber la necesidad de aplicar un tratamiento y analizar la respuesta al mismo.

El método IFI es más sensible que la fijación del complemento para detectar infecciones crónicas, si bien presenta otras limitaciones como la presencia de reacciones cruzadas y la necesidad de una correcta interpretación (experiencia de la persona que interpreta). El test ELISA por su parte presenta ventajas como su sensibilidad para detectar portadores sanos, la posibilidad de analizar un gran número de animales a la vez, pero la detección de los anticuerpos se basa en el reconocimiento de un solo epítopo, y esto limita la sensibilidad cuando se testan variedades de piroplasmas que presentan regiones menos conservadas. Esta limitación ha sido descrita por diferentes autores (Bhoora y col., 2010; Rapoport y col., 2014), y podría ser el motivo de la ausencia de positivos a *B.caballi* usando ELISA comercial en nuestro estudio.

El frotis sigue siendo un buen método sobre todo en periodos febriles, pero se necesita práctica en la lectura de las muestras, es poco útil cuando se deben analizar un gran número de muestras en la población y la baja parasitemia en la piroplasmosis equina es un factor limitante (Wise y col., 2014).

Cuando se pone a punto una prueba molecular, la secuencia de ADN ideal debe presentar una serie de características como una pequeña longitud para contrarrestar problemas derivados de la baja eficiencia de extracción de DNA, baja parasitemia o degradación, y es fundamental su localización en zonas conservadas o genes constitutivos. En nuestro estudio las PCR en tiempo real que utilizamos, fueron las que mostraron la más alta sensibilidad, especificidad y eficiencia de los cebadores, siendo las que detectaron mayor

número de positivos. La secuencia elegida presenta una longitud adecuada y la TaqMan MGB de *B. caballi* consigue reducir problemas asociados a la presencia de mutaciones, evitando problemas asociados a la degradación o fragmentación del DNA. El porcentaje de acuerdo observado entre las cuatro PCRs de *Babesia* y entre las cuatro de *Theileria* fue superior al 90%. Las diferencias entre los positivos detectados por las distintas PCRs no fueron estadísticamente significativos. Sin embargo no todas las muestras positivas a *Babesia* se confirmaron con la secuenciación. La especificidad relativa de las PCRs de *T.equi* se encontraba hacia el 80% y la sensibilidad alrededor del 99%; en *B.caballi* la rSe fue inferior al 50% y la rSp superiror al 93%.

Al comparar las PCRs que se consideraron más adecuadas (PCRs en tiempo real) con las otras pruebas (IFI, ELISA y frotis) se observaron numerosas discordancias en los resultados, algunas justificadas por causas fisiológicas como el periodo desde que el animal se infecta hasta que producen anticuerpos, o la presencia de animales que son solo serológicamente positivos, pero no portadores de piroplasmas. En el caso del frotis, las discordancias en resultados pueden deberse a la baja parasitemia o al hecho que el frotis, a veces no identifica entre géneros de piroplasmas, así pues puede ser positivo al otro género y los estamos analizando erróneamente frente a la especie que se identificó.

La rSe de las técnicas de *B.caballi* que obtuvimos fue discreta (del 0 al 50%), probablemente debido a la baja detección en ELISA, siendo la rSp mucho mayor (>80%). En *T. equi* los valores de rSe fueron netamente superiores, sin embargo los porcentajes de acuerdo observado entre técnicas y entre métodos serológicos fueron algo menores. Se encontraron diferencias significativas dentro de los métodos directos e indirectos, siendo los que mayor número de positivos consiguieron detectar la PCR el tiempo real y el IFI.

A la luz de estos resultados se aconseja combinar una prueba serológica y una molecular independientemente de la técnica. Tan válida puede ser la combinación ELISA-PCR en tiempo real como IFI y PCR.

El estudio de la epidemiología y los factores de riesgo de una enfermedad es muy importante para establecer medidas de control eficaces. La situación endémica se verificó en los estudios 2.1 y 2.2 en los que se calcularon los valores de prevalencia y se evaluaron diferentes factores de riesgo en caballos y asnos de las zonas centrales de Italia y España.

La seroprevalencia fue mayor en Italia que en España para *T.equi* en caballos (39,8% *vs.*

25,2%) y en asnos (54,8% *vs.* 12,25%); en el caso de *B. caballi* fue mayor solo en caballos (8,9% *vs*. 2,2%) ya que los burros italianos resultaron negativos en ELISA, presentando los asnos españoles una prevalencia del 5%. La detección del parásito fue determinada con PCR en los équidos italianos: en los caballos seropositivos fue alta (70,3% *T.equi* y 10,3% *B.caballi)* y en los burros seropositivos a *T.equi* superior al 76%; también se estimó la prevalencia total del parásito para *B.caballi* en los asnos al no encontrarse seropositivos y fue del 5,9%. Con estos datos se ratifica la situación de endemicidad de la piroplasmosis equina en ambas penínsulas.

Los factores de riesgo estudiados estaban relacionados con características individuales, de manejo y ambientales. Casi todos estos factores determinaban la presencia y el contacto con los vectores y han sido revisados recientemente por otros autores (García-Bocanegra y col., 2013; Sumbria y col., 2017). En nuestros estudios se confirmaron factores de riesgo como el género, observándose prevalencias mayores en las hembras por su mayor exposición al vector por vivir en el exterior; la edad, aumentando la prevalencia serológica en animales adultos y ancianos debido a la persistencia de los anticuerpos, en los que sin embargo se observó que la detección por PCR disminuía probablemente debido a la capacidad del animal para controlar la parasitemia. Las razas autóctonas y cruzadas presentaron mayores valores de seroprevalencia y de detección del ADN del parásito debido al diferente sistema de manejo o a la diversa susceptibilidad de las razas. Curiosamente el color de la capa fue otro parámetro significativo, aunque ya se sabía que los animales de capa clara presentaban mayores prevalencias al atraer más las garrapatas. El acceso al pasto o la aptitud mostraron también ser un factor de riesgo determinado por el mayor contacto del animal con los vectores. Otros parámetros estudiados como la altura, suelo, zona climática determinan una mayor presencia de garrapatas ya que están relacionadas con las características ecológicas que favorecen la presencia de las mismas. El uso de tratamientos antiparasitarios se presenta como un factor protector probablemente debido a que los animales desparasitados reciben una atención mayor.

Los estudios filogenéticos desarrollados en el estudio 2.3 han mostrado la presencia de tres clados para cada género, estos resultados han sido observados también por otros autores (Bhoora y col., 2009). En este estudio además se intenta esclarecer la relación con la patogenicidad de estos grupos así como con la presencia de discordancias entre PCR y ELISA y entre PCRs con distinto target (18S y EMA del merozoíto de *T.equi*). En relación a la presencia

de síntomas, en el grupo 1 se encontraban las muestras de la mayoría de los animales sintomáticos, perteneciendo a los grupos 2 y 3 los portadores sanos. En cuanto a la discordancia con resultados ELISA negativo, encontramos los discordantes principalmente en el grupo 1, que es donde se encuentran los sintomáticos, así que esto podría explicar los resultados negativos del ELISA, ya que en los casos de infecciones agudas no hay todavía suficientes anticuerpos específicos. Las discordancias entre la PCR EMA de punto final y anidada utilizadas para realizar el estudio filogenético basado en el 18S se encuentran principalmente en los grupos 2 y 3; con los datos obtenidos es difícil determinar si puede estar relacionado con el límite de detección, la baja parasitemia o la presencia de otros mecanismos génicos del parásito. Estas observaciones sobre las incongruencias entre resultados de serología y PCR y entre métodos moleculares ponen en evidencia la necesidad de profundizar en la biología del parásito, mecanismos de expresión génica y de evasión de la respuesta inmune tal y como indicaron otros autores (Allred, 2001; Kumar y col., 2004). Fueron sorprendentes los hallazgos en las secuencias de las yeguas sospechosas de piroplasmosis y sus abortos (casos presentados en el estudio 2.4) que mostraron una pertenencia a diferentes grupos, siendo difícil establecer si la causa se debe a la coexistencia de diferentes piroplasmas de clados distintos ya que en la secuenciación se obtiene solo la secuencia predominante y se enmascaran las demás aunque en el electroferograma no se detectaron alteraciones; otra explicación puede ser una reinfección con otro tipo de piroplasma durante el periodo que transcurrió desde el aborto hasta que se enviaron las otras muestras.

También se realizó un análisis filogenético usando el target EMA y se obtuvieron tres grupos para *T. equi* si bien no se observó una relación entre los clados resultantes de los dos tipos de análisis filogenético.

Merece especial atención la transmisión transplacentaria que puede causar abortos, piroplasmosis neonatal y también favorece la continuidad de la transmisión del parásito, con potros portadores y seropositivos que difundirán la enfermedad y serán sujetos a restricciones en el comercio.

En el estudio 2.4 se analizaron las muestras de fetos o restos fetales de yeguas con una sintomatología compatible con piroplasmosis y que habían sufrido abortos. Se realizaron pruebas serológicas, moleculares y frotis (sanguíneo e impronta). La serología se confirmó negativa en el feto (Kumar y col., 2008) y en las madres fue positiva a ambos parásitos, en

ELISA e IFI en el primer caso y solo en IFI en la segunda yegua. Las PCR en tiempo real de *B.caballi* fueron positivas en madres y restos fetales, observando el mismo tipo de secuencia mientras que las otras PCR de punto final y anidada para diagnosticar *Babesia* fueron negativas probablemente por la longitud del amplicón y la baja parasitemia frecuentemente observada en estas infecciones. Las PCRs de *Theileria equi* mostraron resultados sorprendentes, ya que se observó en las madres analizadas con la PCR de punto final un resultado negativo (probablemente por un problema de expresión del gen), mientras los fetos eran positivos. En la anidada de piroplásmidos se observaron secuencias pertenecientes a diferentes clados entre madres y fetos, como ya se ha descrito previamente.

Los signos clínicos y las alteraciones hemato-bioquímicas en el curso de la piroplasmosis equina son muy inespecíficas, por ello en el estudio 3 se evaluaron los resultados de análisis sanguíneos de dos grupos (Italia y España) formados por animales sintomáticos, tanto positivos como negativos a esta enfermedad, diagnosticados con IFI y frotis, con el objetivo de encontrar algún parámetro que se pudiera ser biomarcador específico, aunque no se encontró. En ambos grupos se observaron alteraciones comocidas en los animales infectados; disminución de parámetros de la línea roja causada por la hemólisis, trombocitopenia, bilirrubinemia, aumento de las transaminasas e indicadores de daño hepático, de los antioxidantes y radicales libres así como alteraciones en alfa 2 y gamma globulinas, estos resultados concuerdan con otros recientemente publicados (Laus y col., 2015; Mahmoud y col., 2016; Zobba y col., 2008; Barrera y col., 2010). Los animales positivos a *T. equi* del grupo de España presentaban pocos parámetros alterados (solo marcadores hepáticos), lo que hace pensar que probablemente fueran animales solo seropositivos pues como se ha mencionado anteriormente los anticuerpos permanecen tras la infección durante años (de Waal, 1992). Respecto a los valores observados en animales negativos, en el grupo de España se observaron pocas diferencias significativas, probablemente debido al bajo número de positivos. Sin embargo en el grupo italiano con una muestra mayor sí se observaron diferencias significativas principalmente en animales diagnosticados mediante frotis o en positivos a *T.equi* IFI. Muchos parámetros estaban alterados, pero son bastante inespecíficos y otros, aunque significativamente diferentes, estaban dentro del rango. Este hecho nos permite confirmar que por el momento no hay ningún parámetro útil como biomarcador específico de la piroplasmosis.

EPILOGO

Il presente epilogo raccoglie le discussioni presentate in ogni studio.

L'assenza di una prova gold standard e il fatto che le tecniche diagnostiche consigliate dalla OIE siano solo di tipo sierologico, rende difficile l'elezione di un metodo diagnostico quando si debba discriminare tra gli animali solo sierologicamente positivi, portatori sani e animali con infezione acuta dove ancora non si sono sviluppati anticorpi specifici. In questi casi, il solo uso di tecniche sierologiche non permette di differenziare gli animali esposti dei portatori e dei malati e abbiamo bisogno di integrare i test per capire se si debe trattare o se vogliamo analizzare la risposta ad esso.

Il metodo IFI è più sensibile che la fissazione del complemento per individuare infezioni croniche, sebbene presenta altre limitazioni come la presenza di reazioni crociate e una corretta interpretazione (esperienza della persona che interpreta). Il test ELISA dalla sua parte presenta dei vantaggi come la sua sensibilità per l'individuazione di portatori sani, la possibilità di testare un gran numero di animali alla volta, però l'individuazione degli anticorpi si basa nel riconoscimento di un solo epitopo, e questo limita la sensibilità quando si testano varietà di piroplasmi che presentano regioni meno conservate; questo limite è stato descritto da diversi autori (Bhoora et *al*., 2010; Rapoport et *al*., 2014) e potrebbe essere il motivo dell'assenza di positivi a *B.caballi* diagnosticati con l'ELISA nel nostro studio.

Lo striscio sanguigno è tutt'oggi un buon metodo però necessita di pratica nella lettura del campione ed è poco utile quando si devono analizzare un gran numero di campioni nella popolazione; la bassa parassitemia nella piroplasmosi equina è un fattore limitante (Wise et *al.,* 2014).

Quando si mette a punto un test molecolare, il target ideale deve presentare una serie di caratteristiche come essere di piccola longitudine per compensare problemi derivati dalla bassa efficenza di estrazione di DNA, bassa parassitemia o degradazione del DNA, altra caratteristica è essere disegnato su una regione conservata o gene costitutivo. Nel nostro studio le real time adattate da protocolli descritti in letteratura furono quelle che mostrarono la più alta sensibilità, specificità ed efficienza di primer, essendo quelle che hanno individuato un maggior numero di positivi confermati. La seguenza target presenta una longitudine adeguata e la TaqMan MGB di *B.caballi* riesce a ridurre problemi associati alla presenza di

mutazioni, gli ampliconi corti evitano problemi associati alla degradazione e frammentazione del DNA. La percentuale di accordo osservato fra le 4 PCR di *T.equi* si attesta intorno all'80% e la sensibilità all'incirca del 99%; in *B.caballi* la rSe fu inferiore al 50% e la rSp superiore al 93%.

Nel comparare le PCRs che si considerano più adeguate (real time) con altre prove (IFI, ELISA e striscio sanguigno) si osservano numerose discordanze nei risultati, alcune giustificate per cause fisiologiche come la presenza del periodo finestra da che l'animale si infetta fino a che si sviluppano anticorpi, o la presenza di animali che sono solo sierologicamente positivi ma non portatori. Nel caso dello striscio sanguigno , la discordanza nei risultati può essere originata per la bassa parassitemia o per il fatto che lo striscio, a volte non identifica fra generi di piroplasma così può essere positivo ad altri generi diversi a coloro che si stanno analizzando nella nostra statistica.

La rSe della tecnica di *B.caballi* ottenuta fu discreta (da 0 al 50%) probabilmente per la mancata detezione in ELISA ed essendo la rSp molto maggiore (>80%). In *T. equi* i valori di rSe furono nettamente superiori tuttavia la percentuale di accordo osservato fra le tecniche e fra i metodi sierologici furono un po' minori. Sono state trovate differenze significative dentro dei metodi diretti e indiretti. Alla luce di questi risultati, si consiglia di combinare prove sierologiche e molecolari però non si può consigliare né sconsigliare un tipo di tecnica o l'altra.

Lo studio della epidemiologia e i fattori di rischio di una malattia sono molto importanti per stabilire misure di controllo efficaci. Nello studio 2.1 e 2.2. Sono stati calcolati i valori della prevalenza e valutati i differenti fattori di rischio in cavalli e asini delle zone centrali di Italia e Spagna.

La prevalenza fu maggiore in Italia che in Spagna per *T.equi* in cavalli (39,8% contro 25,2%) e in asini (54,8% vs 12,25%) nel caso di *B.caballi* fu maggiore solo in cavalli (8,9% contro 2,2%) poiché gli asini italiani risultarono negativi in ELISA, presentando gli asini spagnoli una prevalenza del 5%; la prevalenza parassitologica determinata con la PCR in cavalli sieropositivi italiani fu alta (70,3% *T.equi* e 10,3% *B.caballi*) e negli asini sieropositivi a *T.equi* superiore al 76%, si stimò anche la prevalenza totale in PCR per *B.caballi* non riscontrando sieropositivi e fu del 5,9%. Con questi dati si ratifica la situazione di endemicità della piroplasmosi equina in ambedue le penisole.

I fattori di rischio studiati sono relazionati con caratteristiche individuali, di maneggio

e ambientali. Questi fattori determinano la presenza e il contatto con i vettori e sono stati descritti da altri autori (García-Bocanegra *et al*., 2013; Sumbria *et al*., 2017). In entrambi gli studi sono stati confermati fattori di rischio come il genere, osservando prevalenze maggiori nelle femmine; l'età , aumentando la prevalenza sierologica in animali adulti e geriatrici dovuta alla persistenza anticorpale. Tuttavia la prevalenza in PCR diminuiva probabilmente per la capacità dell'animale di controllare la parassitemia.

Le razze autoctone e incrociate presentano valori maggiori di prevalenza dovuta al diverso sistema di maneggio o alla diversa suscettibilità delle razze. Il colore del mantello è stato un altro parametro significativo, gli animali dal mantello chiaro presentano maggiore prevalenza poiché attirano di più le zecche. L'accesso al pascolo , é risultato un fattore di rischio determinato dal maggior contatto dell'animale con i vettori. Altri parametri studiati come l'altitudine, il suolo e la zona climatica determinano una maggior presenza di zecche visto che sono relazionate con le caratteristiche ecologiche che favoriscono la loro presenza . L'uso di trattamenti antiparassitari è risultato un fattore protettivo.

Gli studi filogenetici sviluppati nello studio 2.3 hanno mostrato la presenza di tre cladi in ogni genere; questo risultato è stato inoltre osservato da altri autori (Bhoora *et al*., 2009); in questo esperimento si cerca anche di chiarire le relazioni con la patogenicità di questi gruppi così come con la presenza di discordanze in PCR e ELISA e tra PCR con diversi target (18S ed EMA del merozoito di *T.equi*).

In quanto alla presenza di sintomi nel gruppo 1, si raggrupparono i campioni provenienti da animali maggioritariamente sintomatici, riscontrando nel gruppo 2 e 3 i portatori sani; in quanto alla discordanza con risultati ELISA negativi, è stata osservata predominantemente nel gruppo 1, che è dove si trovano i sintomatici, questo giustificherebbe i risultati negativi in sierologia in caso di infezioni acute in cui ancora non si sono sviluppati gli anticorpi specifici. La discordanza fra la PCR end point e la nested utilizzata per fare lo studio filogenetico basato nel 18s, si trova principalmente nei gruppi 2 e 3 ed è difficile determinare se possa essere vincolata al limite di sensibilità, alla bassa parassitemia o alla presenza di altri meccanismi genici del parassita; questa osservazione sull'incongruenza fra risultati di sierologia e PCR e fra metodi molecolari, evidenzia il bisogno di approfondire nella biologia del parassita, nei meccanismi di espressione genica e di evasione della risposta immune (Allred *,* 2001; Kumar *et al.,* 2004).

Inoltre, risultarono sorprendenti i riscontri nelle sequenze delle cavelle sospette di piroplasmosi e i loro aborti (casi presentati nello studio 2.4), che mostrarono una appartenenza a differenti gruppi essendo difficile stabilire se la causa fosse dovuta alla coesistenza di differenti piroplasmi di cladi diversi , poiché il sequenziamento solo mette in evidenza la sequenza più prevalente mascherando le altre (sebbene l'elettroferogramma non aveva detettato alterazioni) o semplicemente fosse stata causata da una reinfezione con un'altro tipo di piroplasma.

È stata realizzata anche un'analisi filogenetica usando il target EMA e sono stati osservati tre gruppi, anche se non è stata stabilita una relazione tra i tre gruppi ottenuti con un'analisi o l'altra.

Merita speciale attenzione la trasmissione transplacentare che può causare aborti, piroplasmosi neonatale e inoltre può favorire la continuità della trasmissione del parassita, con puledri portatori e sieropositivi che diffonderanno la malattia e saranno soggetti a restrizioni nel commercio.

Nello studio 2.4 sono stati analizzati i campioni di feti o le spoglie fetali di fattrici con una sintomatologia compatibile con piroplasmosi e che hanno sofferto aborti. Sono stati testati con prove sierologiche, molecolari e striscio (sanguigno e per apposizione). La sierologia, come si aspettava, nel feto era negativa (Kumar *et al*., 2008) e nelle madri fu positiva ai due parassiti, in ELISA e IFI nel primo caso e solo in IFI nella seconda cavalla. Le PCR real time di *B.caballi* furono positive nelle madri, feto e spoglie fetali, osservando lo stesso tipo di sequenza; le altre PCR usate per diagnosticare *Babesia* furono negative probabilmente per la longitudine dell'amplicone e la bassa parassitemia che si osserva in questa infezione. Le PCR di *Theileria equi* mostrarono risultati sorprendenti, poiché si osservò nelle madri analizzate con l'end point un risultato negativo (probabilmente per un problema di espressione del gene). Nella nested di piroplasmidi, sono state osservate fra madri e feti, sequenze appartenenti a differenti cladi, come descritto previamente.

I segni clinici e le alterazioni emato-biochimiche nel corso della piroplasmosi equina sono molto inespecifiche, per questo nello studio 3 sono stati valutati i risultati delle analisi del sangue di due gruppi (Italia e Spagna) formati da animali sintomatici, positivi e negativi a questa malattia diagnosticata con IFI e striscio sanguigno, con lo scopo di trovare qualche parametro che si possa usare come marcatore specifico. In entrambi i gruppi, si sono osservate

alterazioni simili negli animali infettati; diminuzione dei parametri della serie rossa causata per l'emolisi, trombocitopenia, bilirrubinemia, aumento delle transaminasi e indicatori di danno epatico, antiossidanti e radicali liberi così come alterazioni nelle alfa 2 e gamma globuline. Questi risultati sono in accordo con quelli di altri autori (Laus *et al*., 2015; Mahmoud *et al*., 2016; Zobba et al., 2008; Barrera *et al.,* 2010).

Gli animali positivi a *T.equi* del gruppo spagnolo presentavano pochi parametri alterati (solo marcatori epatici), che fa pensare probabilmente fossero animali solo sieropositivi visto che come menzionato precedentemente, non si ha costanza della sieronegativizzazione (de Waal, 1992). Per quanto riguarda i valori osservati in animali negativi, nel gruppo spagnolo sono state osservate poche differenze significative, probabilmente dovuto al basso numero di positivi, tuttavia nel gruppo italiano sono state osservate differenze significative in diversi parametri principalmente in animali diagnosticati mediante striscio o in positivi a *T.equi* IFI, molti di questi parametri risultavano alterati , ma sono parametri abbastanza inespecifici; altri parametri risultarono significativi ma rimanevano sempre dentro al range. Questo fatto non ci ha permesso di stabilire nessun parametro utile come marcatore diagnostico della malattia.

CONCLUSIONES

CONCLUSIONS **CONCLUSIONI**

1. La comparación de las diferentes técnicas de diagnóstico disponibles demuestra que es necesario complementar el uso de pruebas serológicas, siendo de elección el inmunodiagnóstico, con métodos moleculares sensibles y específicos basados en la amplificación de secuencias preferiblemente de corta longitud presentes en regiones conservadas o constitutivas para establecer un correcto diagnóstico que nos permita diferenciar entre animales seropositivos, portadores asintomáticos y animales que presentan infecciones agudas y que aún no han seroconvertido.

2. Las prevalencias encontradas en caballos y asnos de zonas centrales de la Península Ibérica e Itálica confirman la situación endémica en ambas áreas observándose mayores seroprevalencias para *Theileria equi* que para *Babesia caballi* en ambos países y siendo los valores encontrados en Italia superiores a los de España.

3. La evidente influencia de los diferentes factores de riesgo estudiados y asociados al manejo de los équidos y a las condiciones ambientales de las áreas de muestreo que favorecen la presencia e interacción con los vectores indican la necesidad de implementar programas de control de vectores y mejorar las medidas de manejo de los animales.

4. Los resultados de los estudios filogenéticos muestran la presencia de distintos clados, tres para *Babesia* y tres para *Theileria;* con los que se establecen diferencias de patogenicidad entre variantes y falsos negativos a otras pruebas diagnósticas como la PCR o el ELISA lo que evidencia la necesidad de profundizar en la biología del parásito y sus mecanismos de expresión génica.

5. La presentación clínica de los casos en ambos países es similar, observándose las mismas variaciones en los parámetros sanguíneos estudiados. No se ha determinado ningún parámetro hematológico ni bioquímico útil como biomarcador de enfermedad.

6. Se confirma la transmisión vertical de *Theileria equi* y de *Babesia caballi (*ésta última nunca antes evidenciada en Europa) en dos yeguas de razas autóctonas italianas, poniendo de manifiesto la importancia de la transmisión intrauterina en la piroplasmosis equina que produce abortos, mortalidad neonatal causada por formas agudas de piroplasmosis en los potros y favorece la propagación de la enfermedad.

EN

CONCLUSIONS

1. Comparison of the different available diagnostic techniques demonstrates the need to complement the use of serological tests (immunodiagnosis is the better choice), with specific and sensitive molecular methods based on the amplification of short length sequences present in conserved or constitutive regions to establish a correct diagnosis which allows to differentiate among seropositive animals, asymptomatic carriers and animals presenting acute infections which have not yet seroconverted.

2. The prevalences found in horses and donkeys from central areas in the Iberian and Italian Peninsulas confirm the endemic situation in both regions; showing higher seroprevalences for *Theileria equi* than for *Babesia caballi* in both countries with higher values in Italy.

3. The evident influence of the different studied risk factors which are associated with the equids management and the environmental conditions in the sampling areas that favor the presence and interaction with the vectors indicate the need to implement vector control programs and to improve animals management

IT

CONCLUSIONI

1. La comparazione tra diverse tecniche diagnostiche disponibili, dimostra la necessità di integrare l'uso di test sierologici, con preferenza per l'immunodiagnostico, con metodi molecolari sensibili e specifici basati nell'amplificazione di sequenze preferibilmente di corta longitudine presenti in regioni conservate o costitutive, per stabilire una corretta diagnosi che permetta di distingure tra animali sieropositivi, portatori asintomatici e animali che presentano infezioni acute e che non hanno ancora sieroconvertito.

2. Le prevalenze riscontrate in cavalli e asini in zone centrali della penisola Iberica e Italica, confermano la situazione endemica in entrambe le aree, osservando maggiori sieroprevalenze per *Theileria equi* che per *Babesia Caballi* in entrambi i paesi, essendo i valori riscontrati in Italia superiori a quelli riscontrati in Spagna.

3. L'evidente influenza dei diversi fattori di rischio studiati e associati al maneggio degli equidi e alle condizioni ambientali delle aree di campionamento che favoriscono la presenza e interazione con i vettori, evidenziano la necessità di

measures.

4. The results of the phylogenetic studies demonstrate the presence of different clades, three for *Babesia* and three for *Theileria*; showing differences in pathogenicity among variants and false negatives results to other diagnostic tests such as PCR or ELISA, which confirm the need to deepen the biology of the parasite and its mechanisms of gene expression.

5. Similar clinical presentation in both countries is observed, showing the same variations in the studied blood parameters. No hematological or biochemical parameters have been determined as a disease biomarker.

6. Vertical transmission of *Theileria equi* and *Babesia caballi* (the latter never evidenced before in Europe) in two Italian autochthonous bred mares is confirmed; highlighting the importance of intrauterine transmission in equine piroplasmosis which causes abortions, neonatal mortality due to acute forms of piroplasmosis in foals and promotes the spread of the disease.

implementare i programmi di controllo dei vettori e migliorare le misure di maneggio degli animali.

4. I risultati degli studi filogenetici mostrano la presenza di diversi cladi, tre per *Babesia* e tre per *Theileria;* Con questi risultati, si stabiliscono differenze di patogenicità tra stipiti e falsi negativi ad altre prove diagnostiche come la PCR o l'ELISA, che evidenzia la necessità di approfondire nella biologia del parassita e sui suoi meccanismi di espressione genica.

5. La presentazione clinica dei casi in entrambi i paesi è simile, riscontrando le stesse variazioni nei parametri sanguigni studiati. Non si è determinato nessun parametro ematologico né biochimico utile come biomarcatore della malattia.

6. Si conferma la trasmissione verticale di *Theileria equi e Babesia caballi (*quest'ultima mai evidenziata prima in Europa) in due cavalle di razze autoctone italiane, mettendo in rilievo l'importanza della trasmissione intrauterina nella piroplasmosi equina che produce aborti, mortalità neonatale causata da forme acute di piroplasmosi nei puledri e favorisce la propagazione della malattia.

RESUMEN

La piroplasmosis equina es una enfermedad presente en la lista de la OIE causada por *Babesia caballi* y *Theileria equi* que afecta a équidos y es transmitida por garrapatas ixódidas. Puede presentarse enforma aguda, subaguda o crónica. Su distribución es mundial y genera importantes pérdidas económicas directas e indirectas relacionadas con la importación de animales.

En la primera parte del estudio se procedió a evaluar diferentes técnicas diagnósticas, ya que las que prescribe la OIE son solo serológicas, para ello se pusieron a punto diferentes protocolos moleculares y se determinó el más sensible, se calcularon la sensibilidad y especificidad relativas y el porcentaje de acuerdo observado entre métodos, no solo moleculares sino serológicos y frotis, llegando a la conclusión de que es necesario unir varias técnicas diagnósticas en los análisis rutinarios.

En la segunda parte del estudio se realizaron estudios de prevalencia en zonas centrales de España e Italia confirmándose el estado de zona endémica; también se analizaron diferentes factores de riesgo de tipo ambiental y de manejo, observándose diferencias estadísticamente significativas.

Asimismo se desarrolló un estudio filogenético de los parásitos evidenciados en PCR, construyéndose árboles filogenéticos y observando la presencia de tres grupos para cada género; se establecieron relaciones de diversa patogenicidad entre grupos y negatividad a otros métodos moleculares.

Otro estudio llevado a cabo en esta parte tiene como objeto el análisis de muestras procedentes de yeguas con síntomas atribuidos a la piroplasmosis y que habían sufrido aborto, procediendo también a analizar el feto o material fetal mediante distintas técnicas serológicas, moleculares y frotis observando el paso por vía vertical de ambos géneros de protozoos.

En la última parte de la tesis se describe un estudio en el que se analizaron muestras de animales sintomáticos en los laboratorios de los centros donde se ha desarrollado la presente tesis en Italia y España, para valorar las diferencias entre animales positivos y negativos y poder determinar un marcador biológico. Se valoraron diferentes parámetros hemato-bioquímicos y se procedió a diagnosticar la enfermedad mediante IFI y frotis, las variaciones en las variables responden a los cambios ya descritos por otros autores en el curso de la piroplasmosis y se individuaron diferencias estadísticamente significativas entre animales

positivos y negativos, pero no se determinó ningún biomarcador útil.

SUMMARY

Equine piroplasmosis, caused by *Babesia caballi* and *Theileria equi*, is a disease present on the OIE list that affects equids and is transmitted by ixodide ticks. It can appear as an acute, subacute or chronic stage. It has a worldwide distribution and generates significant direct and indirect economic losses related to the importation of animals.

In the first part of the study different diagnostic techniques were evaluated. Since those prescribed by the OIE are only serological, different molecular protocols were developed and the most sensitive was determined. The relative sensitivity and specificity were calculated as well as the percentage of agreement between methods, not only molecular but serological and smears, concluding that it is necessary to join several diagnostic techniques in the routine analyzes.

In the second part of the study, prevalence studies were carried out in central areas of Spain and Italy, confirming the endemic zone status. Different environmental and management risk factors were also analyzed, with statistically significant differences being observed. A phylogenetic study of the parasites was developed and evidenced by PCR. By the construction of phylogenetic trees and observing the presence of three groups for each genre relationships of different pathogenicity between groups and negativity to other molecular methods were established.

Another study carried out in this part analyzed samples from symptomatic mares that had undergone abortion. The fetus and placental material was also analyzed using different serological, molecular and smear techniques observing the vertical transmission of both genres of protozoa.

The last part of the thesis presents a study in which several samples of symptomatic animals were analyzed in the laboratories of the centers where the present thesis was developed in Italy and Spain, in order to assess differences between positive and negative animals and to be able to determine a biological marker of disease. Different hemato-biochemical parameters were evaluated and the disease was diagnosed by IFAT and blood smears. Variations in the variables respond to the changes already described by other authors during the course of piroplasmosis, and statistically significant differences were identified between positive and negative animals. There was not determined a useful biomarker for the disease.

RIASSUNTO

La piroplasmosi equina è una malattia presente nella lista della OIE causata da *Babesia caballi* e *Theileria equi* che infetta equini ed è trasmessa tramite zecche Ixodidae e può presentare forma acuta, subacuta e cronica. La sua distribuzione è mondiale e causa importanti perdite economiche direttamente e indirettamente relazionate con l'importazione degli animali.

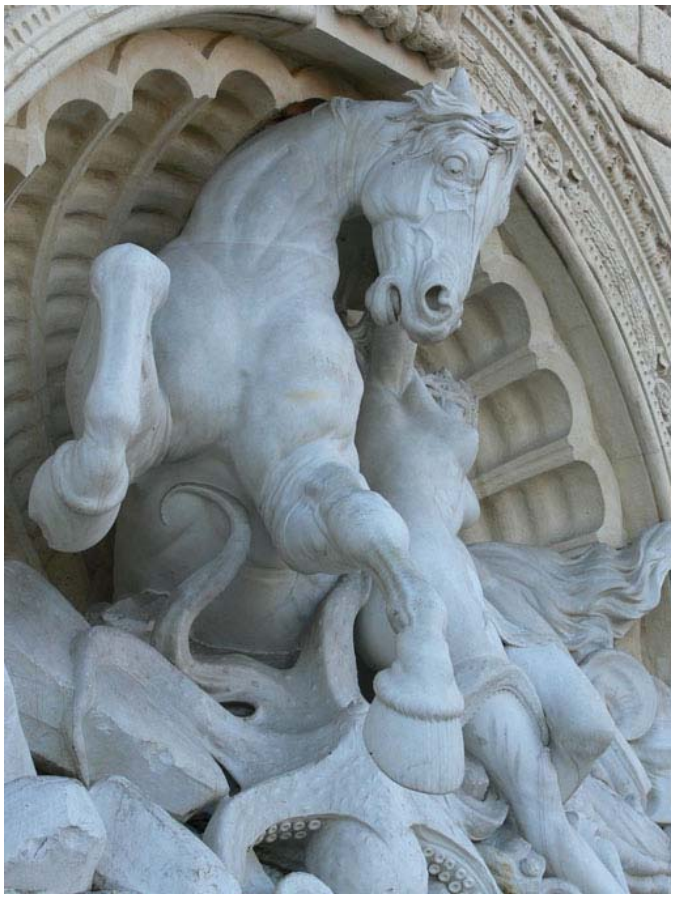
Nella prima parte dello studio sono state valutate differenti tecniche diagnostiche, visto che quelle raccomandate dalla OIE sono solo sierologiche. A questo scopo sono stati messi a punto differenti protocolli molecolari e sono state determinati i più sensibili, sono state calcolate la sensibilità relativa, la specificità relativa e la percentuale di accordo osservato fra i metodi, non solo molecolari ma anche sierologiche e degli strisci sanguigni, arrivando alla conclusione che è necessario integrare diverse tecniche diagnostiche nelle analisi di routine.

Nella seconda parte dello studio, sono stati svolti studi di prevalenza in zone centrali di Spagna e Italia confermando la situazione di zona endemica in entrambe i paesi; inoltre sono stati analizzati diversi fattori di rischio di tipo ambientale e di maneggio, osservando differenze statistiche significative.

Allo stesso tempo si sviluppò uno studio filogenetico dei parassiti evidenziati in PCR, costruendo alberi filogenetici e osservando la presenza di tre gruppi per genere. Sono state stabilite relazioni di diversa patogenicità tra i gruppi e negatività ad altri metodi molecolari.

Un altro studio effettuato in questo capitolo, ha come obiettivo l'analisi dei campioni di fattrici con sintomatologia compatibile con piroplasmosi e che avevano abortito; sono stati analizzati feto e spoglie fetali utilizzando tecniche sierologiche, molecolari e di striscio sanguigno confermando la trasmissione per via verticale di ambedue i generi di protozoi.

Nell'ultima parte della tesi si presenta uno studio in cui, sono stati analizzati campioni di animali sintomatici, nei laboratori dei centri dove è stata sviluppata la presente tesi in Italia e Spagna, per osservare le differenze tra animali positivi e negativi in modo tale di poter determinare un marcatore biologico. Sono stati valutati differenti parametri emato-biochimici e si è diagnosticata la malattia mediante IFI e striscio sanguigno, le variazioni ematobiochimiche determinate sono simili a quelle descritte in corso di piroplasmosi da altri autori; sono state osservate differenze statisticamente significative tra animali negativi e positivi, ma non è stato determinato nessun biomarcatore utile.



BIBLIOGRAFÍA / REFERENCES

Abutarbush, S. M.; Alqawasmeh, D. M.; Mukbel, R. M.; Al-Majali, A. M. Equine Babesiosis: Seroprevalence, Risk Factors and Comparison of Different Diagnostic Methods in Jordan. *Transbound. Emerg. Dis.* **2012**, *59*, 72–78.2.

Aharonson-Raz, K.; Rapoport, A.; Hawari, I. M.; Lensky, I. M.; Berlin, D.; Zivotofsky, D.; Klement, E.; Steinman, A. Novel description of force of infection and risk factors associated with *Theileria equi* in horses in Israel and in The Palestinian Authority. *Ticks Tick. Borne. Dis.* **2014**, *5*, 366–372.3.

Al-Saad.*,* Acute Babesiosis in Foals**.** *J. Animal Veterinary Adv**. 2009.*** 8, 12, 2585–2589.

Almarza N., Sobre la diferenciación de *Piroplasma caballi* y *Nuttalia equi*: A propósito del primer caso de Nuttaliosis del caballo diagnosticado en España Ganadería, **1933**; I (1), 24-37

Allred, D. R. Molecular technology and antigenic variation among intraerythrocytic hemoparasites: Do we see reality? *Vet. Parasitol.* **2001**, *101*, 261–274.

Allsopp, M. T. E. P.; Lewis, B. D.; Penzhorn, B. L. Molecular evidence for transplacental transmission of *Theileria equi* from carrier mares to their apparently healthy foals. *Vet. Parasitol.* **2007**, *148*, 130–136.2.

Ambawat, H. K.; Malhotra, D. V.; Kumar, S.; Dhar, S. Erythrocyte associated haemato-biochemical changes in *Babesia equi* infection experimentally produced in donkeys. *Vet. Parasitol.* **1999**, *85*, 319–324.5.

Babes, V. Sur l'hemoglobinurie bacterienne du boeuf. *Compt. Rend. Acad. Sci. Ser. III Sci. Vie*. **1888**. 107, 692–694.

Barrera, R.; Carapeto, M. V.; Habela, M. A.; Zaragoza, C. Electrophoretic pattern of serum proteins in horses with babesiosis. *Arch. Med. Vet.* **2010**, *42*, 173–178.3.

Bartolomé del Pino, L. E.; Nardini R.; Veneziano V.; Iacoponi F.; Cersini A., Autorino G.L., Buono F.; Scicluna M.T. *Babesia caballi* and *Theileria equi* infections in horses in Central-Southern Italy: Sero-molecular survey and associated risk factors. *Ticks Tick. Borne. Dis.* **2016**, *7*, 462–469.4.

Bashiruddin, J. B.; Cammà, C.; Rebêlo, E. Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. *Vet. Parasitol.*

1999, *84*, 75–83.5.

Beck, R.; Vojta, L.; Mrljak, V.; Marinculić, A.; Beck, A.; Živičnjak, T.; Cacciò, S. M. Diversity of *Babesia* and *Theileria* species in symptomatic and asymptomatic dogs in Croatia. *Int. J. Parasitol.* **2009**, *39*, 843–848.6.

Bhoora, R.; Franssen, L.; Oosthuizen, M. C.; Guthrie, A. J.; Zweygarth, E.; Penzhorn, B. L.; Jongejan, F.; Collins, N. E. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. *Vet. Parasitol.* **2009**, *159*, 112–120.7.

Bhoora, R.; Quan, M.; Franssen, L.; Butler, C. M.; Van der Kolk, J. H.; Guthrie, A. J.; Zweygarth, E.; Jongejan, F.; Collins, N. E. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. *Vet. Parasitol.* **2010**, *168*, 201–211.8.

Bhoora, R.; Quan, M.; Zweygarth, E.; Guthrie, A. J.; Prinsloo, S. a.; Collins, N. E. Sequence heterogeneity in the gene encoding the rhoptry-associated protein-1 (RAP-1) of *Babesia caballi* isolates from South Africa. *Vet. Parasitol.* **2010**, *169*, 279–288.10.

Bhoora, R.; Quan, M.; Matjila, P. T.; Zweygarth, E.; Guthrie, A. J.; Collins, N. E. Sequence heterogeneity in the equi merozoite antigen gene (ema-1) of *Theileria equi* and development of an ema-1-specific TaqMan MGB assay for the detection of *T. equi*. *Vet. Parasitol.* **2010**, *172*, 33–45.9.

Bhoora, R.; Buss, P.; Guthrie, A. J.; Penzhorn, B. L.; Collins, N. E. Genetic diversity of piroplasms in plains zebra (*Equus quagga burchellii*) and Cape mountain zebra (*Equus zebra zebra*) in South Africa. *Vet. Parasitol.* **2010**, *174*, 145–149.6.

Brüning, A. Equine piroplasmosis an update on diagnosis, treatment and prevention. *Br. Vet. J.* **1996**, *152*, 139–151.11.

Brüning, A.; Phipps, P.; Posnett, E.; Canning, E. U. Monoclonal antibodies against *Babesia caballi* and *Babesia equi* and their application in serodiagnosis. *Vet. Parasitol.* **1997**, *68*, 11–26.12.

Butler, C. M.; Sloet van Oldruitenborgh-Oosterbaan, M. M.; Stout, T. A. E.; van der Kolk, J. H.; van den Wollenberg, L.; Nielen, M.; Jongejan, F.; Werners, A. H.; Houwers, D. J.

Prevalence of the causative agents of equine piroplasmosis in the South West of The Netherlands and the identification of two autochthonous clinical *Theileria equi* infections. *Vet. J.* **2012**, *193*, 381–385.13.

Cacciò, S.; Cammà, C.; Onuma, M.; Severini, C. The β-tubulin gene of *Babesia* and *Theileria* parasites is an informative marker for species discrimination. *Int. J. Parasitol.* **2000**, *30*, 1181–1185.14.

Camacho, A. T.; Guitian, F. J.; Pallas, E.; Gestal, J. J.; Olmeda, A. S.; Habela, M. A.; Telford, S. R.; Spielman, A. *Theileria (Babesia) equi* and *Babesia caballi* infections in horses in Galicia, Spain. *Trop. Anim. Health Prod.* **2005**, *37*, 293–302.15.

Coleto L. Equine babesiosis: A disease linked to the extensive horse raising in the pastureland of Extremadura "dehesa" In Etienne M. *Cahiers Options Méditerranées*, **1999**, n. 39; p. 273-276.

Criado-Fornelio, A.; Martinez-Marcos, a.; Buling-Saraña, a.; Barba-Carretero, J. C. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe: Part I. Epizootiological aspects. *Vet. Parasitol.* **2003**, *113*, 189–201.19.

Criado-Fornelio, A.; Martinez-Marcos, A.; Buling-Saraña, A.; Barba-Carretero, J. C. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe: Part II. Phylogenetic analysis and evolutionary history. *Vet. Parasitol.* **2003**, *114*, 173–194.18.

Criado-Fornelio, A.; Gónzalez-Del-Río, M. A.; Buling-Saraña, A.; Barba-Carretero, J. C. The "expanding universe" of piroplasms. *Vet. Parasitol.* **2004**, *119*, 337–345.17.

Criado, A.; Martinez, J.; Buling, a.; Barba, J. C.; Merino, S.; Jefferies, R.; Irwin, P. J. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. *Vet. Parasitol.* **2006**, *142*, 238–247.16.

de Waal, D. T. Equine piroplasmosis: a review. *Br. Vet. J.* **1992**, *148*, 6–14.20.

de Waal D.T.; Heerden J. Equine piroplasmosis. In: Coetzer JAW, Tustin RC, editors. Infectious diseases of livestock with special reference to South Africa. Vol 1. Oxford University Press; **1994**. 293–304.

de Waal D.T.; Heerden J. Equine piroplasmosis. In: Coetzer JAW, Tustin RC, editors. Infectious diseases of livestock. 2. New York: Oxford University Press; **2004**. pp. 425–433.

Deger, S.; Deger, Y.; Bicek, K.; Ozdal, N.; Gul, A. Status of Lipid Peroxidation, Antioxidants, and Oxidation Products of Nitric Oxide in Equine Babesiosis: Status of Antioxidant and Oxidant in Equine Babesiosis. *J. Equine Vet. Sci.* **2009**, *29*, 743–747.8.

Diana, A.; Guglielmini, C.; Candini, D.; Pietra, M.; Cipone, M. Cardiac arrhythmias associated with piroplasmosis in the horse: A case report. *Vet. J.* **2007**, *174*, 193–195.21

Friedhoff KT; Soule C.; Li A; An account on equine babesioses. *Rev. Sci. Tech.* **1986,** 15:1191-201.

Fritz, D. A PCR study of piroplasms in 166 dogs and 111 horses in France (March 2006 to March 2008). *Parasitol. Res.* **2010**, *106*, 1339–1342.24.

García-Bocanegra, I.; Arenas-Montes, A.; Hernández, E.; Adaszek, Ł.; Carbonero, A.; Almería, S.; Jaén-Téllez, J. A.; Gutiérrez-Palomino, P.; Arenas, A. Seroprevalence and risk factors associated with *Babesia caballi* and *Theileria equi* infection in equids. *Vet. J.* **2013**, *195*, 172–178.23.

Georges, K. C.; Ezeokoli, C. D.; Sparagano, O.; Pargass, I.; Campbell, M.; D'Abadie, R.; Yabsley, M. J. A case of transplacental transmission of *Theileria equi* in a foal in Trinidad. *Vet. Parasitol.* **2011**, *175*, 363–366.25.

Grandi, G., Molinari, G., Tittarelli, M., Sassera D., Kramer L., 2011 Prevalence of *Theileria equi* and *Babesia caballi* infection in horses from Northern Italy. *Vector Borne Zoonotic Dis*. **2011.** 11, 955-956.

Guglielmi G. Un caso di malaria nel cavallo. *La Clinica Vet*. **1899**; pp. 220-223.

Guidi, E.; Pradier, S.; Lebert, I.; Leblond, A. Piroplasmosis in an endemic area: analysis of the risk factors and their implications in the control of Theileriosis and Babesiosis in horses. *Parasitol. Res.* **2015**, *114*, 71–83.9.

Habela M., Reina D.; Nieto, C; Verdugo SG, Navarrete I y col.: «Epidemiología de la babesiosis equina en Extremadura: Estudio preliminar». *Med. Vet.,* **1989;** 6: 31-39.

Habela, A, Sevilla, G., Corchero, E., Pe, J., Extremadura, S. Diagnóstico ytratamiento de la Piroplasmosis equina. *Mundo Vet.* **2000**, noviembre, 62–68.

Hailat, N. Q.; Lafi, S. Q.; Al-Darraji, A. M.; Al-Ani, F. K. Equine babesiosis associated with strenuous exercise: Clinical and pathological studies in Jordan. *Vet. Parasitol.* **1997**, *69*,

1–8.26.

Hanafusa, Y.; Cho, K. O.; Kanemaru, T.; Wada, R.; Sugimoto, C.; Onuma, M. Pathogenesis of *Babesia caballi* infection in experimental horses. *J. Vet. Med. Sci.* **1998**, *60*, 1127–32.27.

Hines, S. A.; Ramsay, J. D.; Kappmeyer, L. S.; Lau, A. O.; Ojo, K. K.; Van Voorhis, W. C.; Knowles, D. P.; Mealey, R. H. *Theileria equi* isolates vary in susceptibility to imidocarb dipropionate but demonstrate uniform in vitro susceptibility to a bumped kinase inhibitor. *Parasit. Vectors* **2015**, *8*, 33.10.

Holman, P. J.; Hietala, S. K.; Kayashima, L. R.; Olson, D.; Waghela, S. D.; Wagner, G. G. Case report: Field-acquired subclinical *Babesia equi* infection confirmed by in vitro culture. *J. Clin. Microbiol.* **1997**, *35*, 474–476.11.

Inci A. Detection of *Babesia caballi* (Nuttall, 1901) and *Babesia equi* (Laveran 1901) in horses by microscopic examination in Military Farm in Gemlik. *Turk. J. Vet. Anim. Sci*. 1997. 21, 43–46.

Iori, A.; Gabrielli, S.; Calderini, P.; Moretti, A.; Pietrobelli, M.; Tampieri, M. P.; Galuppi, R.; Cancrini, G. Tick reservoirs for piroplasms in central and northern Italy. *Vet. Parasitol.* **2010**, *170*, 291–296.12.

Karatepe, B.; Karatepe, M.; Çakmak, A.; Karaer, Z.; Ergün, G. Investigation of seroprevalence of *Theileria equi* and *Babesia caballi* in horses in Nigde province, Turkey. *Trop. Anim. Health Prod.* **2009**, *41*, 109–113.13.

Ketter-Ratzon, D.; Tirosh-Levy, S.; Nachum-Biala, Y.; Saar, T.; Qura, L.; Zivotofsky, D.; Abdeen, Z.; Baneth, G.; Steinman, A. Accepted. *Ticks Tick. Borne. Dis.* **2017**.28.

Koch R. Beitra È ge zur entwicklungsgeschichte der piroplasmen. *Z Hyg. Infektionskrankh*. **1906**; 54:9

Kouam, M. K.; Kantzoura, V.; Gajadhar, A. A.; Theis, J. H.; Papadopoulos, E.; Theodoropoulos Georgios, G. Seroprevalence of equine piroplasms and host-related factors associated with infection in Greece. *Vet. Parasitol.* **2010**, *169*, 273–278.14.

Kumar, S.; Malhotra, D. V.; Dhar, S.; Nichani, A. K. Vaccination of donkeys against *Babesia equi* using killed merozoite immunogen. *Vet. Parasitol.* **2002**, *106*, 19–33.29.

Kumar, S.; Yokoyama, N.; Kim, J. Y.; Huang, X.; Inoue, N.; Xuan, X.; Igarashi, I.; Sugimoto, C.

Expression of *Babesia equi* EMA-1 and EMA-2 during merozoite developmental stages in erythrocyte and their interaction with erythrocytic membrane skeleton. *Mol. Biochem. Parasitol.* **2004**, *133*, 221–227.31.

Kumar, S.; Malhotra, D. V.; Sangwan, A. K.; Goel, P.; Kumar, A.; Kumar, S. Infectivity rate and transmission potential of *Hyalomma anatolicum anatolicum* ticks for *Babesia equi* infection. *Vet. Parasitol.* **2007**, *144*, 338–343.32.

Kumar, S.; Kumar, R.; Gupta, a. K.; Dwivedi, S. K. Passive transfer of *Theileria equi* antibodies to neonate foals of immune tolerant mares. *Vet. Parasitol.* **2008**, *151*, 80–85.

Kumar, S.; Kumar, R.; Sugimoto, C. A perspective on *Theileria equi* infections in donkeys. *Jpn. J. Vet. Res.* **2009**, *56*, 171–180.30.

Laus, F.; Veronesi, F.; Passamonti, F.; Paggi, E.; Cerquetella, M.; Hyatt, D.; Tesei, B.; Fioretti, D. P. Prevalence of tick borne pathogens in horses from Italy. *J. Vet. Med. Sci.* **2013**, *75*, 715–20.15.

Laus, F.; Spaterna, A.; Faillace, V.; Veronesi, F.; Ravagnan, S.; Beribé, F.; Cerquetella, M.; Meligrana, M.; Tesei, B. Clinical investigation on *Theileria equi* and *Babesia caballi* infections in Italian donkeys. *BMC Vet. Res.* **2015**, *11*, 100.33.

Laveran A. Contribution to the study of *Piroplasma equi*. CR Seances Soc Biol Paris **1901**; 53:385-6.

Mahmoud, M. S.; El-Ezz, N. T. A.; Abdel-Shafy, S.; Nassar, S. A.; El Namaky, A. H.; Khalil, W. K. B.; Knowles, D.; Kappmeyer, L.; Silva, M. G.; Suarez, C. E. Assessment of *Theileria equi* and *Babesia caballi* infections in equine populations in Egypt by molecular, serological and hematological approaches. *Parasit. Vectors* **2016**, *9*, 260.34.

Mahoney DF; Wright IG; Frerichs WM; Groenendyk S; O'Sullivan BM; Roberts MC; The identification of *B. equi* in Australia. Waddell AH. *Aust Vet J* **1977.** Oct; 53 (10):461-4.

Mantran, A.; Votion, D. M.; Amory, H.; Introduction, I. Piroplasmose : une réalité belge. **2004**.

Mehlhorn, H.; Schein, E. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. *Parasitol. Res.* **1998**, *84*, 467–475.35.

Metcalf, E. S. The role of international transport of equine semen on disease transmission. *Anim. Reprod. Sci.* **2001**, *68*, 229–237.36.

Moretti, A.; Mangili, V.; Salvatori, R.; Maresca, C.; Scoccia, E.; Torina, A.; Moretta, I.; Gabrielli, S.; Tampieri, M. P.; Pietrobelli, M. Prevalence and diagnosis of *Babesia* and *Theileria* infections in horses in Italy: A preliminary study. *Vet. J.* **2010**, *184*, 346–350.16.

Munkhjargal, T.; Sivakumar, T.; Battsetseg, B.; Nyamjargal, T.; Aboulaila, M.; Purevtseren, B.; Bayarsaikhan, D.; Byambaa, B.; Terkawi, M. A.; Yokoyama, N.; Igarashi, I. Prevalence and genetic diversity of equine piroplasms in Tov province, Mongolia. *Infect. Genet. Evol.* **2013**, *16*, 178–185.37.

Nagore, D.; García-Sanmartín, J.; García-Pérez, A. L.; Juste, R. a.; Hurtado, A. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: Epidemiological survey and phylogenetic analysis. *Vet. Parasitol.* **2004**, *123*, 41–54.38.

Nuttall G.H.F.; Strickland. A.; The parasites of the horse piroplasmosis response of biliary fever. *Zentralb Bakteriol.* **1910**; 1:524-5

Okamura, M.; Yokoyama, N.; Wickramathilaka, N. P. A. L. K.; Takabatake, N.; Ikehara, Y.; Igarashi, I. *Babesia caballi* and *Babesia equi*: Implications of host sialic acids in erythrocyte infection. *Exp. Parasitol.* **2005**, *110*, 406–411.39.

Olmeda, **A.S**., Bösse R., Ndjeng P., Sanmartín H., Meana A., Babesiosis equina: serodetección por inmunofluorescencia en animales sintomáticos y clínicamente sanos. V Simposium ibérico sobre ixodoidea y enfermedades transmitidas. Madrid 27-29 septiembre de **2000**: 79-80

Pfeifer Barbosa, I.; Böse, R.; Peymann, B.; Friedhoff, K. T. Epidemiological aspects of equine babesioses in a herd of horses in Brazil. *Vet. Parasitol.* **1995**, *58*, 1–8.17. Phipps, L. P. Equine Piroplasmosis. *Equine Vet Edu* **1996**, *8*, 33–36.18.

Phipps L.P. Equine piroplasmosis*. Equine vet. Educ***. 1996**. 8. 1.33-36

Qablan, M. A.; Sloboda, M.; Jirků, M.; Oborník, M.; Dwairi, S.; Amr, Z. S.; Hořín, P.; Lukeš, J.; Modrý, D. Quest for the piroplasms in camels: Identification of *Theileria equi* and *Babesia caballi* in Jordanian dromedaries by PCR. *Vet. Parasitol.* **2012**, *186*, 456–460.40.

Rapoport, A.; Aharonson-Raz, K.; Berlin, D.; Tal, S.; Gottlieb, Y.; Klement, E.; Steinman, A. Molecular characterization of the *Babesia caballi* rap-1 gene and epidemiological survey in horses in Israel. *Infect. Genet. Evol.* **2014**, *23*, 115–120.41.

Rhalem, A; Sahibi, H.; Lasri, S.; Johnson, W. C.; Kappmeyer, L. S.; Hamidouch, a; Knowles, D. P.; Goff, W. L. Validation of a competitive enzyme-linked immunosorbent assay for diagnosing *Babesia equi* infections of Moroccan origin and its use in determining the seroprevalence of *B. equi* in Morocco. *J. Vet. Diagn. Invest.* **2001**, *13*, 249–251.19.

Ribeiro, M. F. B.; Passos, L. M. F. Natural co-infection of *Babesia caballi* and *Encephalitozoon-like* microsporidia in the tick *Anocentor nitens* (Acari: Ixodidae). *J. Invertebr. Pathol.* **2006**, *93*, 183–185.42.

Ros-García, A.; M'ghirbi, Y.; Hurtado, A.; Bouattour, A. Prevalence and genetic diversity of piroplasm species in horses and ticks from Tunisia. *Infect. Genet. Evol.* **2013**, *17*, 33–37.20.

Rothschild, C. M. Equine piroplasmosis. *J. Equine Vet. Sci.* **2013**, *33*, 497–508.21.

Rubino, G.; Cito, A. M.; Lacinio, R.; Bramante, G.; Caroli, A.; Pieragostini, E.; Petazzi, F. Hematology and some blood chemical parameters as a function of tick-borne disease (TBD) signs in horses. *J. Equine Vet. Sci.* **2006**, *26*, 475–480.44.

Sant, C.; d'Abadie, R.; Pargass, I.; Basu, A. K.; Asgarali, Z.; Charles, R. A.; Georges, K. C. Prospective study investigating transplacental transmission of equine piroplasmosis in thoroughbred foals in Trinidad. *Vet. Parasitol.* **2016**, *226*, 132–137.45.

Savini G., Battistini ML, Scaramozzino P, Tittarelli M, Molteni C. Le babesiosi equine. La situazione italiana. *Summa.* **1997***,* 4, 35-40.

Scoles, G.; **Ueti, M. W**. Vector Ecology of Equine Piroplasmosis. *Annu. Rev. Entomol.* **2015**, *60*, 561–580.46.

Serra, J.M.S.; Fonseca, I.M.P.; Carvalho, L.M.M.; Varela, M.C. Estudo das parasitoses dos equídeos do Ribatejo: II Babesioses. *Acta Parasitológica Portuguesa*, **1993**,1**.**

Sevinc, F.; Maden, M.; Kumas, C.; Sevinc, M.; Ekici, O. D. A comparative study on the prevalence of *Theileria equi* and *Babesia caballi* infections in horse sub-populations in Turkey. *Vet. Parasitol.* **2008**, *156*, 173–177.22.

Shkap, V.; Cohen, I.; Leibovitz, B.; Savitsky; Pipano, E.; Avni, G.; Shofer, S.; Giger, U.; Kappmeyer, L.; Knowles, D. Seroprevalence of *Babesia equi* among horses in Israel using competitive inhibition ELISA and IFA assays. *Vet. Parasitol.* **1998**, *76*, 251–259.23.

Singh, B.; Banerjee, D. P.; Gautam, O. P. Comparative efficacy of diminazene diaceturate and imidocarb dipropionate against *Babesia equi* infection in donkeys. *Vet. Parasitol.* **1980**, *7*, 173–179.24.

Sippel W.L., Cooperrider D.E., Gainer J.H., Allen R.W., Mouw J.E.B., Tiegland M.B. Equine piroplasmosis in the United States. *J Am Vet Med Assoc*. **1962**, 141:694–698.

Soulé C; Perret, O. F. J. D. P. Répartition géographique des babésioses équines latentes en France.*Méd Mal Infec.t* **1998**, *28*, 403–404.25.

Steinman, A.; Zimmerman, T.; Klement, E.; Lensky, I. M.; Berlin, D.; Gottlieb, Y.; Baneth, G. Demographic and environmental risk factors for infection by *Theileria equi* in 590 horses in Israel. *Vet. Parasitol.* **2012**, *187*, 558–562.

Sumbria, D.; Singla, L. D.; Sharma, A.; Bal, M. S.; Randhawa, C. S. Molecular survey in relation to risk factors and haemato-biochemical alteration in *Theileria equi* infection of equines in Punjab Province, India. *Vet. Parasitol. Reg. Stud. Reports* **2017**, *8*, 43–50.48.

Wise, L. N.; Pelzel-McCluskey, A. M.; Knowles, D. P. Equine Piroplasmosis. *Vet. Clin. North Am. Equine Pract.* **2014**, *30*, 677–693.

Zanet S.; Bassano, M.; Trisciuoglio, A.; Taricco, I.; Ferroglio, E. Horses infected by Piroplasms different from *Babesia caballi* and *Theileria equi*: species identification and risk factors analysis in Italy. *Vet. Parasitol.* **2017.**

Zobba, R.; Ardu, M.; Niccolini, S.; Chessa, B.; Manna, L.; Cocco, R.; Pinna Parpaglia, M. L. Clinical and Laboratory Findings in Equine Piroplasmosis. *J. Equine Vet. Sci.* **2008,** *28*, 301–308.

ANEXO I. Características de las PCR utilizadas

APPENDIX I. Characteristics of the PCRs used **ANNESSO I. Caratteristiche delle PCR utilizzate**

END POINT PCR *BABESIA CABALLI*
(Bhoora *et al.*, 2010)

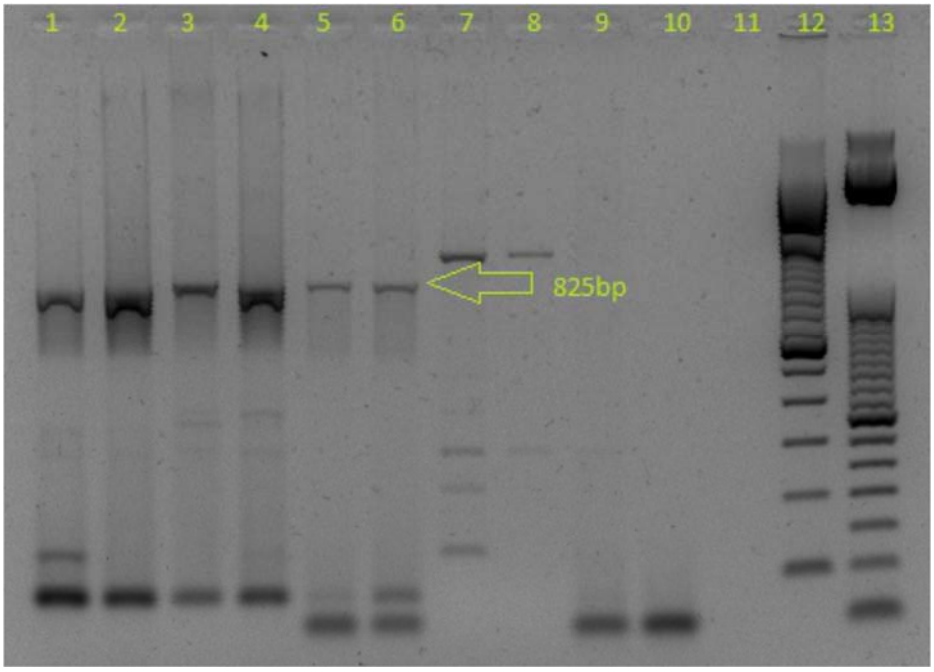
Master Mix Kit: Gold Taq Polymerase kit (Applied Biosystems)

Reagents	Volume for 1 sample	Final concentrations
H_2O-DEPC	33.84µL	
Buffer (10X)	5µl	1X
BC-RAP2F 30µM	0,83µl	0,5 µM
BC-RAP2R 30µM	0,83µl	0,5 µM
dNTP Mix 10mM	1µl	0,2mM
$MgCl_2$ 25mM	3µl	1.5mM
Ampli TaqGold 5U/µl	0,5 µl	0,05U/ µl
DNA	5 µl	
Total Volume	50µl	

95°C for 10 minutes
45 cycles: 95°C for 60 seconds, 58°C for 60 seconds, 72°C for 60 seconds
72°C for 7 minutes

Product: 825 bp

Detection limit: *not specified* in the article



NESTED PCR *BABESIA CABALLI*
(Battsetseg *et al*., 2002)

Master Mix Kit: Gold Taq Polymerase kit (Applied Biosystems)

PCR1 BC48

Reagents	Volume for 1 sample	Final concentrations
H_2O-DEPC	33.59µL	
Buffer (10X)	5µL	(1X)
BC48F1 30µM	0,83µL	0,5µM
BC48R3 30µM	0,83µL	0,5µM
dNTP Mix 10mM	1.25µL	0,25mM
$MgCl_2$ 25mM	3µL	1.5mM
Ampli Taq Gold 5U/µL	0,5µL	0,05U/µL
DNA	5µL	
Total Volume	50µL	

95°C for 10 minutes
40 cycles: 94°C for 60 seconds, 60°C for 2 minutes, 72°C for 60 seconds
72°C for 7 minutes
Product: 530 bp

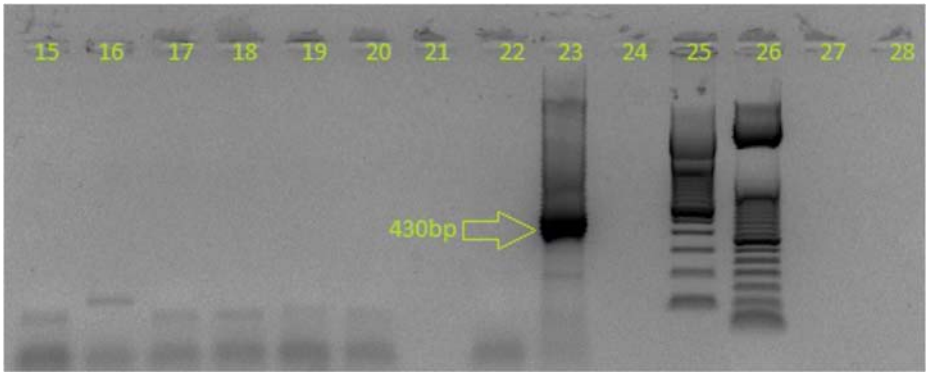
PCR2 BC48

Reagents	Volume for 1 sample	Final concentrations
H_2O-DEPC	33.59µL	
Buffer (10X)	5µL	(1X)
BC48_F11 30µM	0,83µL	0,5µM
BC48_R31 30µM	0,83µL	0,5µM
dNTP Mix 10mM	1.25µL	0,25mM
$MgCl_2$ 25mM	3µL	1.5mM
Ampli Taq Gold (5U/µL)	0,5µL	0,05U/µL
DNA	5µL	
Total Volume	50µL	

95°C for 10 minutes
40 cycles: 94°C for 60 seconds, 60°C for 2 minutes, 72°C for 60 seconds
72°C for 7 minutes

Product: 430 bp.

Detection limit: 0.01pg/µL of DNA



REAL TIME *BABESIA CABALLI*
(Bhoora *et al.*, 2010)

Probe: FAM-NFQ (Sonda MGB)

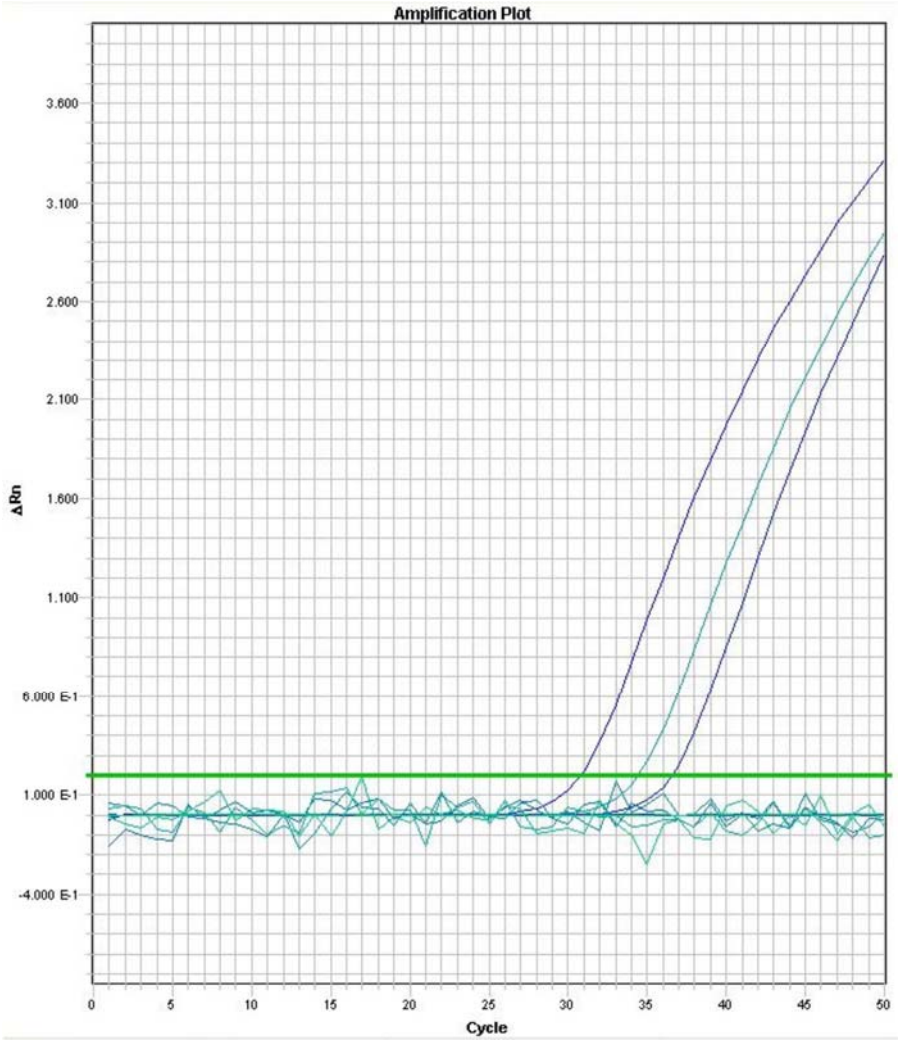
Master Mix Kit: TaqMan Universal PCR Master Mix (Applied Biosystems)

Reagents	Volume for 1 sample	Final concentrations
H_2O G.R.	5,37µL	
TaqMan 2X PCR Master Mix	12,5µL	(1X)
Primer BC18SF402 30µM	0,75µL	0,9µM
Primer BC18SR496 30µM	0,75µL	0,9µM
Probe BC-18SP 10mM	0,63µL	0,25mM
DNA	5µL	
Total Volume	25µL	

50°C for 2 minutes
95°C for 10 minutes
50 cycles: 95°C 20 seconds and 60°C for 1 minute

Product: 101bp.

Detection limit: 1.14x 10^{-4} parasited erythrocytes



NESTED PCR PIROPLASMS
(Nagore *et al.*, 2004)

Master Mix Kit: Gold TaqPolymerasi KIt (Applied Biosystems)

PCR1 RLB

Reagents	Volume for 1 sample	Final concentrations
H_2O G.R.	15.61µl	
Buffer (10X)	2.5µl	1X
MgCl2 50mM	0.75µl	1.5mM
dNTP 10mM	0.5µl	0.2mM
Primer RLB F1. 30µM	0.17µl	0.2µM
Primer RLB R1. 30µM	0.17µl	0.2µM
Taq Polymerasi 5U/µl	0.3µl	0.06U/µl
DNA	5µl	
Total Volume	25µl	

94°C for 5 minutes
40 cycles: 94°C for 35 seconds, 51°C for 35 seconds, 72°C for 35 seconds
72°C for 10 minutes
Product: 460bp for *T.equi* and 520bp for *B. caballi.*

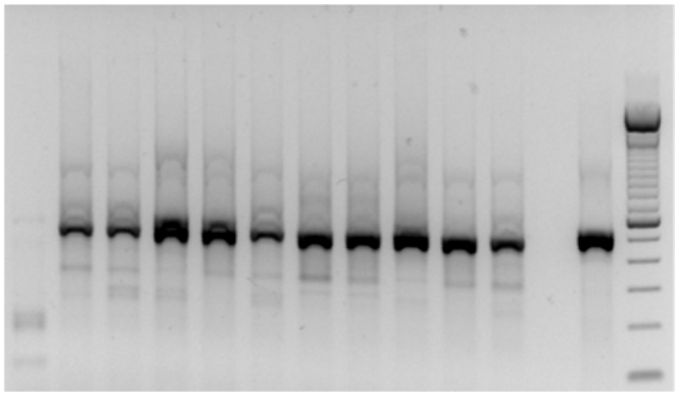
PCR2 RLB 2

Reagents	Volume for 1 sample	Final concentrations
H_2O G.R.	14.09µl	
Buffer (10X)	2.5µl	1X
MgCl2 50mM	0.75µl	1.5mM
dNTP 10mM	0.5µl	0.2mM
Primer RLB F2 30µM	0.83µl	1µM
Primer RLB R2 30µM	0.83µl	1µM
Taq Polymerasi 5U/µl	0.5µl	0.10U/µl
DNA	5µl	
Total Volume	25µl	

94°C for 5 minutes
40 cycles: 94°C for 60 seconds, 54°C for 60 seconds, 72°C for 1.5 minutes
72°C for 10 minutes

Product: 430bp for *T.equi* and 390bp for *B. caballi.*

Detection limit: 10^2 to 10^3 copies.



END POINT PCR *THEILERIA EQUI*
(Battsetseg *et al.,* 2002)

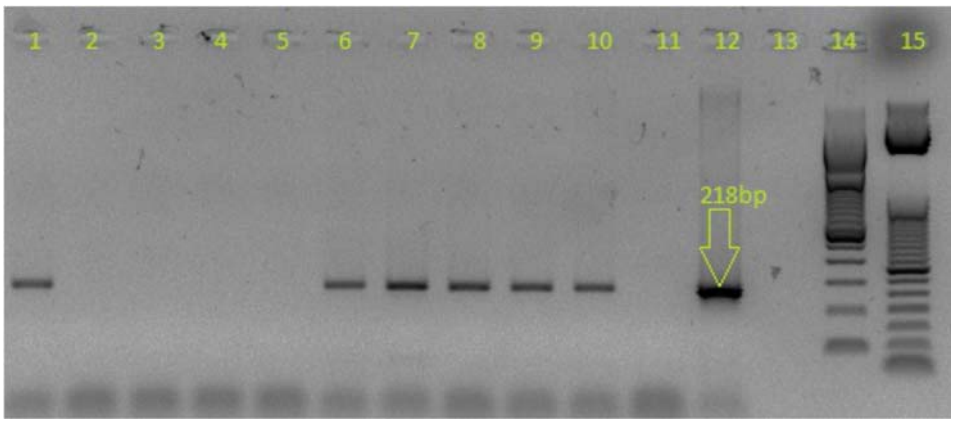
Master Mix Kit: Gold TaqPolymerasi KIt (Applied Biosystems)

Reagents	Volume for 1 sample	Final concentrations
H_2O G.R.	31.6µl	
Buffer (10X)	5µl	1X
MgCl2 25mM	3µl	1.5mM
dNTP 10mM	1.25µl	0.25mM
BSA 1mg/ml	2.5µL	50µg/ml
Primer EMA5. 30µM	0.7µl	0.4µM
Primer EMA6 .30µM	0.7µl	0.4µM
AmpliTaq Gold 5U/µl	0.25µl	0.025U/µl
DNA	5µl	
Total Volume	50µl	

94°C for 10 minutes
35 cycles: 94°C for 60 seconds, 60°C for 60 seconds, 72°C for 60 seconds
72°C for 10 minutes

Product: 268 pb

Detection limit: 0.01pg/µL of DNA



NESTED PCR *THEILERIA EQUI*
(Nicolaiewsky *et al*., 2001)

Master Mix Kit: Gold Taq Polymerase kit (Applied Biosystems)

PCR1 EMAE T.EQUI

Reagents	Volume for 1 sample	Final concentrations
H_2O-GR	33.59µL	
Buffer (10X)	5µL	1X
EMAE-F 30µM	0.83µL	0,5µM
EMAE-R 30µM	0.83µL	0,5µM
dNTP Mix 10mM	1.25µL	0,25mM
$MgCl_2$ 25mM	3µL	1.5mM
Ampli TaqGold 5U/µL	0,5µL	0,05U/µL
DNA	5µL	
Total Volume	50µL	

94°C for 10 minutes
40 cycles: 94°C for 40 seconds, 60°C for 60 seconds, 72°C for 60 seconds
72°C for 4 minutes
Product: 369 pb

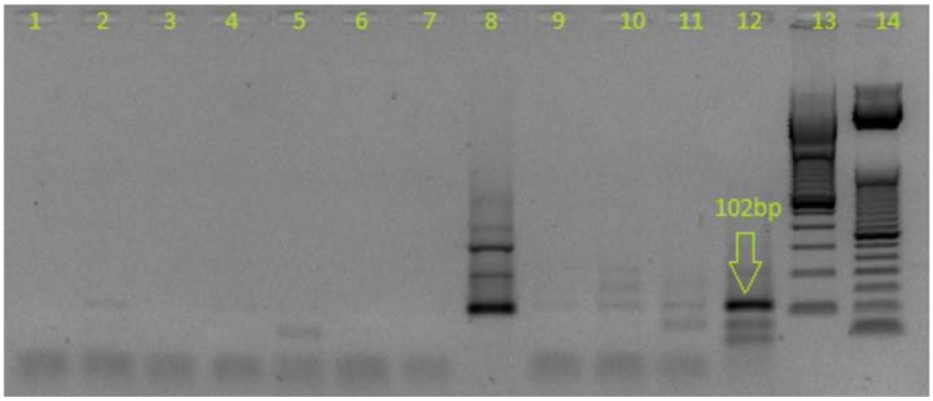
PCR2 EMAI T.EQUI

Reagents	Volume for 1 sample	Final concentrations
H_2O-GR	33.59µL	
Buffer (10X)	5µL	1X
EMAI-F 30µM	0.83µL	0,5µM
EMAI-R 30µM	0.83µL	0,5µM
dNTP Mix 10mM	1.25µL	0,25mM
$MgCl_2$ 25mM	3µL	1.5mM
Ampli TaqGold (5U/µL)	0,5µL	0,05U/µL
DNA	5µL	
Total Volume	50µL	

94°C for 10 minutes
35 cycles: 94°C for 60 seconds, 60°C for 45 seconds, 72°C for 45 seconds
72°C for 5 minutes

Product: 102 pb

Detection limit: 6 infected cells on 10^8 total red cells.



REAL *TIME THEILERIA EQUI*
(Kim *et al.*, 2008)
Probe: VIC - TAMRA

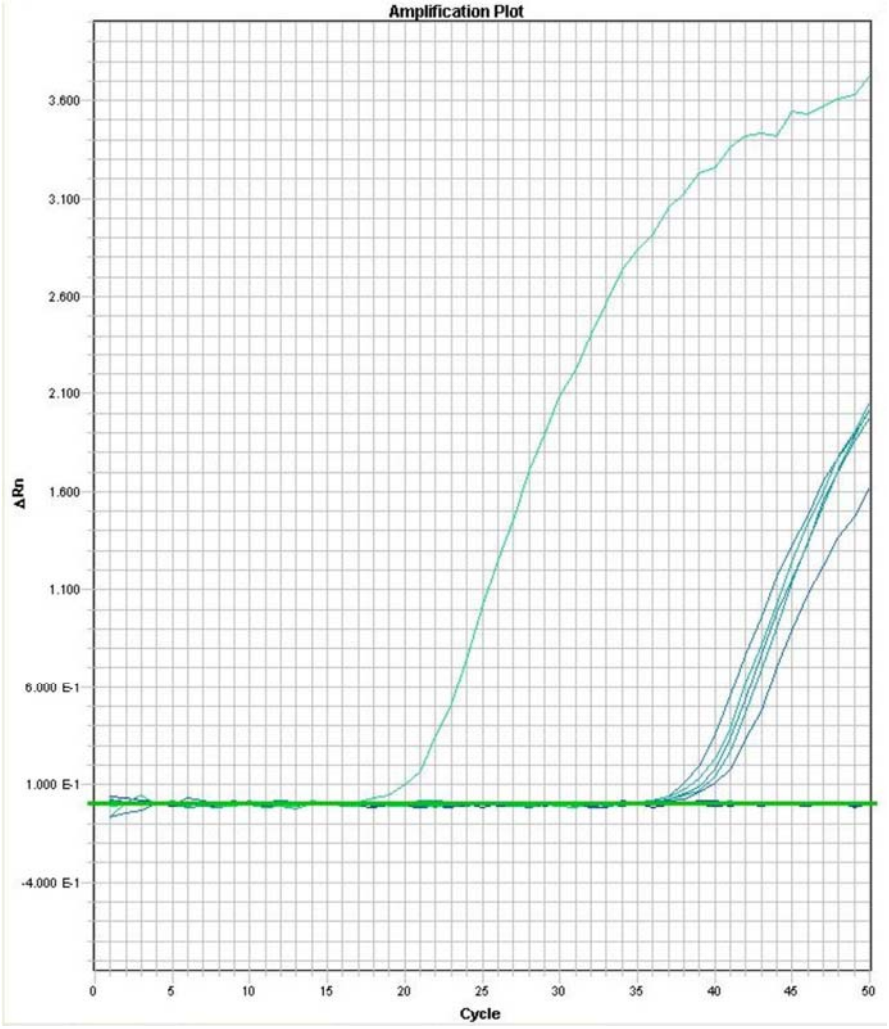
Master Mix Kit: TaqMan Universal PCR Master Mix (Applied Biosystems)

Reagents	Volume for 1 sample	Final concentrations
H_2O G.R.	5.37µL	
TaqMan 2XPCR Master Mix	12.5µL	1X
Primer Be18SF 30µM	0.75µL	0.9µM
Primer Be18SR 30µM	0.75µL	0.9µM
Probe Be18SP 10mM	0.63µL	0.25µM
DNA	5µL	
Total Volume	25µL	

50°C for 2 minutes
95°C for 10 minutes
50 cycles: 95°C 20 seconds and 55°C for 1 minute

Product: 81bp.

Detection limit: 1.9 x 10^{-4} parasited erythrocytes



ANEXO II. Abreviaturas

APPENDIX II. Abbrevations **ANNESSO II. Abbreviazioni**

ADN: Ácido desoxirribonucleico
ALB: Albumin
ALPHA 1: Alpha 1 globulins
ALPHA 2: Alpha 2 globulins
A/G: Ratio albumin/globulins
BAND NEU: Band neutrophils
BASO: Basophils
BETA: Beta globulins
B. caballi: Babesia caballi
BUN: Blood urea nitrogen
CD4+: T-helper lymphocytes
CD8+: Cytotoxic T lymphocytes
CD4+/CD8+: ratio of T-helper cells to cytotoxic T cells
CK: Creatine kinase
CERME: National Reference Centre for Equid Diseases
CREA: Creatinine
Ct: Threshold cycle.
DIRECT BIL: Direct bilirubin
DNA: Deoxyribonucleic acid
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
EP: equine piroplasmosis
EOS: Eosinophils
GAMMA: Gamma globulins
GGT: Gamma glutamyltransferase
GPT: Glutamate-pyruvate transaminase
HCT: Hematocrit
HGB: Hemoglobin
IFAT: Immunofluorescence antibody test
IFI: Inmunoflurescencia indirecta
IFN: Interferons
IL: Interleukin
INDIRECT BIL: Indirect bilirubin
IZSLT: Istituto Zooprofilattico delle Regioni Lazio e Toscana
LDH: Lactate dehydrogenase
LYM: Lymphocytes
MCH: Mean corpuscular hemoglobin
MCHC: Mean corpuscular hemoglobin concentration
MONO: Monocytes
MPV: Mean platelet volume
NEU: Neutrophils
NO: Nitric oxide
OIE: World Organisation for Animal Health
PCT: Plateletcrit
PCR: Polymerase Chain Reaction
PDW: Plate volume distribution width
PLT: Platelet
RBC: Red blood cells
RDW: Red blood cell volume distribution width

T. equi: Theileria equi
TNF: Tumoral necrosis factor
TOT BIL: Total bilirubin
TOTAL PROT: Total proteins
UCM: Universidad Complutense de Madrid
Vs: Versus
WBC: White blood cells

ANEXO III: Publicaciones y presentaciones a congresos

APPENDIX III. Publications and conference presentations

ANNESSO III. Publicazioni e presentazioni a convegni

-*Babesia caballi* and *Theileria equi* infections in horses in Central-Southern Italy: Sero-molecular survey and associated risk factors Ticks and tick-borne diseases 7(3):462–469 January .2016 Leticia E Bartolome del Pino, Roberto Nardini, Vincenzo Veneziano, Francesca Iacoponi, Antonella Cersini, Gian Luca Autorino, Francesco Buono, Maria Teresa Scicluna.

-Preliminary investigations on the sequence heterogeneity of the 18S rRNA gene of *Theileria equi* and *Babesia caballi* strains collected from a horse population in Central Italy Journal of Equine Veterinary Science 39 S45eS55 April 2016. Antonella Cersini, Leticia E Bartolome del Pino, Valeria Antognetti, Raniero Lorenzetti, Roberto Nardini, Gian Luca Autorino, Maria Teresa Scicluna

-Preliminary results on the inclusion of PCR for the diagnosis of equine piroplasmosis (EP) Journal of Equine Veterinary Science 39 (2016) S7eS19 April 2016 Maria Teresa Scicluna, Roberto Nardini, Leticia E Bartolome del Pino, Antonella Cersini, Giuseppe Manna, Gian Luca Autorino

-Prevalence of equine piroplasmosis in central Spain. Leticia E. Bartolomé del Pino, Miguel Llorens Picher, Aránzazu Meana Mañes. Poster presentation for the XXIX National Congress *SoIPa* (Società Italiana di Parassitologia) & *European*Veterinary *Parasitology* College. Bari (Italy), June 2016.

-Addition of PCR methods to the conventional serology for the routine diagnosis of equine piroplasmosis. Scicluna MT, Nardini R, Bartolomé del Pino LE, Ricci I, Rosone F, Autorino GL. Oral presentation for the Congress of the *World Association of Veterinary Laboratory Diagnosticians* (WAVLD) Saskatoon-Canada (June 2015).

-Preliminary observations on the genetic heterogeneity of *Theileria equi* and *Babesia caballi* in the horse population of central Italia. Cersini A, Scicluna MT, Bartolomé del Pino LE, Nardini R, Conti R, Manna G, Autorino GL. Poster presentation for the congress of the *World Association of Veterinary Laboratory Diagnosticians* (WAVLD) Saskatoon-Canada (June 2015).

-Evaluation of PCR methods for the molecular detection of *Babesia caballi* and *Theileria equi* on field samples**.** Bartolomé del Pino L.E., Cersini A., Scicluna M.T., Nardini R., Manna G., Antognetti V., Autorino G.L. Oral presentation for the congress of the *European Association of Veterinary Laboratory Diagnosticians* (EAVLD) held in Pisa-Italy (October 2014). Presenter and attendee thanks to a grant offered by the Organization.

Ticks and Tick-borne Diseases 7 (2016) 462–469

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Babesia caballi and *Theileria equi* infections in horses in Central-Southern Italy: Sero-molecular survey and associated risk factors

Bartolomé Del Pino Leticia Elisa [a], Nardini Roberto [b], Veneziano Vincenzo [c], Iacoponi Francesca [b], Cersini Antonella [b], Autorino Gian Luca [b], Buono Francesco [c], Scicluna Maria Teresa [b,*]

[a] *Complutense University of Madrid, Madrid, Spain*
[b] *National Reference Centre for Equine Diseases, Istituto Zooprofilattico Sperimentale "M. Aleandri", Via Appia Nuova 1411, 00178 Rome, Italy*
[c] *Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Via F. Delpino, 1 80137, Naples, Italy*

ARTICLE INFO

Article history:
Received 4 September 2015
Received in revised form 12 January 2016
Accepted 19 January 2016
Available online 21 January 2016

Keywords:
Theileria equi
Babesia caballi
Italy
Horse
sero-molecular prevalence
Risk-factor

ABSTRACT

Babesia caballi and *Theileria equi* are tick-borne pathogens, etiological agents of equine piroplasmosis that affect different species of Equidae causing relevantly important direct and indirect losses.

A field study was conducted to evaluate the distribution of the equine piroplasms in an area of Central-Southern Italy and to identify correlated risk factors. Serum samples of 673 asymptomatic horses were collected during spring-summer of 2013 to estimate the seroprevalence of the parasites within the study area using *T. equi* and *B. caballi* Antibody test kit (VMRD®, Inc, Pullman, WA, USA). The 273 seropositive samples were subsequently tested by real time PCR to verify the presence of the genome of the piroplasms, indicative of the carrier status of the subjects. The variables chosen to identify which were the risk factors associated with the serological and PCR-positivity for each of the equine piroplasms were the following: gender, age, breed, access to pasture, altitude, land cover, climatic zone, soil type and province location (coastal/inland).

The resulting overall seroprevalence for *T. equi* was 39.8% (268/673) and for *B. caballi* was 8.9% (60/673) while 70.3% of the PCR tested samples (185/263) were positive for *T. equi* and 10.3% (27/263) for *B. caballi*. The univariate and multiple logistic regression models were used to assess the association of the risk factors with the different outcomes. The risk factors found to be associated with *T. equi* seropositivity were gender, age, breed, access to pasture, land cover, soil type and province location, while those associated with PCR-positivity were age, soil type and province location. As the number of *B. caballi* seropositive subjects was limited, the multiple logistic regression model was performed only for the PCR-positive status, identifying climatic zone and soil type as the sole risk factors. In the study area, a major diffusion of *T. equi*, in terms of seroprevalence and PCR-positivity was present when compared to that of *B. caballi*, probably related to the cumulative effect of the life-long infection of the former protozoan. The identification of risk factors relative to each piroplasm infection, specific to a study area, is important in the development and improvement of tailored control and prevention programmes aimed at containing health and economic consequences.

© 2016 Elsevier GmbH. All rights reserved.

1. Introduction

Equine piroplasmosis (EP) is a disease caused by two species of intra-erythrocytic protozoa, namely *Babesia caballi* and *Theileria equi* that affects horses, mules, donkeys and zebras. Both parasites are transmitted by ticks of genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus* (Scoles and Ueti, 2015). EP is endemic in tropical and temperate areas and occurs in acute, sub acute and chronic forms. Typical clinical signs of EP are fever, depression, anaemia, icterus, oedema, anorexia and, occasionally, mucosal petechiae and ecchymoses. Horses surviving the acute phase may remain seropositive, inapparent carriers with low levels of parasitaemia, condition that occurs more frequently in *T. equi* infections (De Waal, 1992). While disease due to *B. caballi* is reported as less severe than that induced

* Corresponding author. Tel.: +39 06 79099 315; fax: +39 06 79340724.
E-mail address: teresa.scicluna@izslt.it (S.M. Teresa).

http://dx.doi.org/10.1016/j.ttbdis.2016.01.011
1877-959X/© 2016 Elsevier GmbH. All rights reserved.

by *T. equi*, clinical signs are common to both protozoa (De Waal, 1992). For this, differential diagnosis on clinical basis is unreliable and is therefore performed using laboratory methods represented by stained blood smears, serological tests, such as complement fixation test, indirect fluorescent antibody test (IFAT), ELISA, and PCR methods (Sumbria et al., 2015). EP is a major constraint to the international movement of horses causing important economical losses to the horse industry (Friedhoff et al., 1990). Prevalence studies conducted in other areas of Italy reported different levels of seropositivity when using IFAT, ranging from 0.3% (Grandi et al., 2011) to 56.0% (Moretti et al., 2010) for *B. caballi* and from 8.2% (Grandi et al., 2011) to 50.5% (Moretti et al., 2010) for *T. equi*. Using PCR, different levels of positivity were also described, ranging from 0% (Grandi et al., 2011) to 6.0% (Laus et al., 2013) for *B. caballi* and from 11.7% (Laus et al., 2013) to 33.0% (Grandi et al., 2011) for *T. equi*. The aims of this paper were to determine the prevalence of both parasites, serologically and using PCR assays in asymptomatic horses of Central-Southern Italy and to identify the associated risk factors, not yet investigated for the specific area and species.

2. Materials and methods

2.1. Study area and sampling method

This study involved the horse population of an area of Central-Southern Italy as represented in Fig. 1. Sample size was defined on an expected prevalence of 50% of an infinite population, a confidence interval of 95% (95% CI) and an absolute accuracy of 5% that resulted in 384 samples. Although other studies report higher equine piroplasmosis prevalence levels (Moretti et al., 2010), the sample size definition criteria were selected to maximise the accuracy of the prevalence estimation. Qualified veterinarians randomly collected blood samples, with and without EDTA, during spring-summer 2013 from the long-term resident horse population of the study area. The serum was obtained by centrifugation for 10 min at 358 g and stored at −20 °C while, uncoagulated blood was stored at −80 °C. All operations on the horses were performed with the owner's consent and according to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

To identify equine piroplasm related risk factors, data on the following variables were registered at blood collection: gender (gelding; male; female); age (young ≤ 6 years; adult between 7 and 12 years; senior > 12 years); breed (foreign breed; Italian breed; mixed breed); access to pasture (yes/no) province location of stable (coastal/inland). Furthermore, using the Global Positioning System, the geographic location of the animals included in the study was established allowing other variables to be evaluated. These were altitude (≤150 meters above sea level (m asl); 151–600 m asl; > 600 m asl); land cover (> 75% forest; crops 50–75%; 50–75% forest; mixed, with no dominant land cover); climatic zone, based on length of growing period (LGP) which is number of days during a

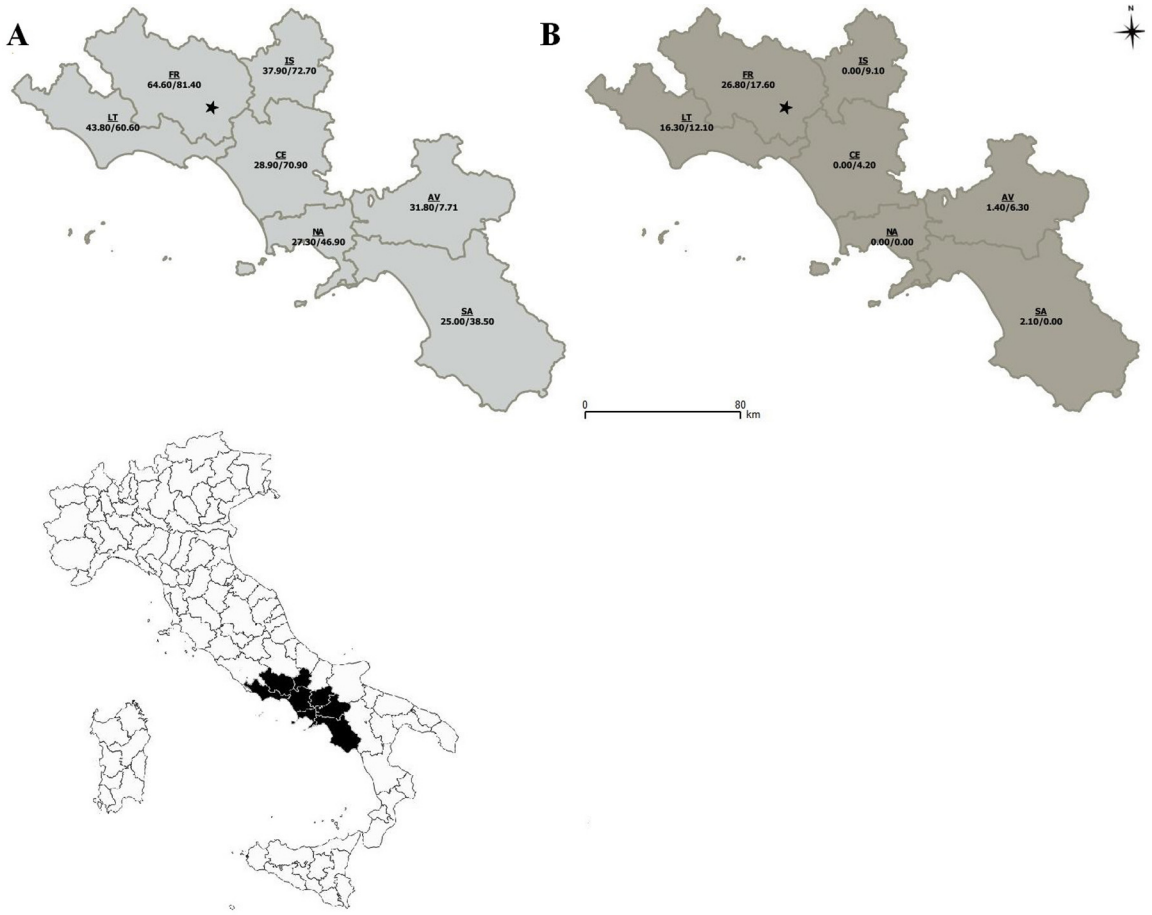


Fig. 1. Serological and PCR-positivity prevalences for *T. equi* (A) and *B. caballi* (B) for each province investigated. First number is the serological prevalence, second number is the PCR-positivity prevalence. FR = Frosinone and LT = Latina belong to Latium Region; IS = Isernia to Molise Region; AV = Avellino; CE = Caserta; NA = Naples and SA = Salerno to Campania Region. CE, LT, NA, SA are coastal provinces, the others are inland. Province location in Italy is shown at bottom left. ⋆ in figure represents location of Aurunci Mountains.

year, when precipitation exceeds half the potential evapotranspiration (humid LGP 270-365 days; sub-humid LGP 180-269 days; moist-semiarid LGP 120-179 days); soil type, for which, referring to the Food and Agricultural Organization (FAO) classification, the types identified in the study area were eutric cambisol, district cambisol, andosol and chromic luvisol. The eutric cambisols are among the most productive soils while the dystric cambisols, although less fertile, are used for mixed arable farming and grazing lands. Andosols are intensively cultivated and are planted with a variety of crops, even if their major limitation is rendering phosphorus unavailable to plants. Chromic luvisols are rich in iron hydroxides with a high nutrient content and a good drainage, usually forming flat or gently sloping landscapes, under climatic regimes that range from cool temperate to warm Mediterranean. Their characteristics make them suitable for a wide range of cultures, from grains to orchards to vineyards.

The FAO website[1] was used to obtain information about land cover, climatic zone and soil type relative to the horses' geographic location.

Details on the classification of the climatic zones can be obtained from the FAO website[2] while those for soil types from the International Soil Reference and Information Centre (ISRIC) – World Soil Information[3] and the FAO website[4].

2.2. Serological tests

Two commercial competitive ELISA (cELISA) *B. equi* Antibody test kit and *B. caballi* Antibody test kit (VMRD®, Inc, Pullman, WA, USA) were employed according to manufacturers instructions.

2.3. Molecular tests

As seropositive animals in an asymptomatic population are not indicative of a recent or active infection, the EDTA blood of the 273 seropositve animals was examined for PCR-positivity to identify those with a double reactivity (serological and molecular) that could better correlate with a recent/active infection and identify the risk factors associated with this status.

The Real Time PCR (rtPCR) protocols employed were those described by Kim et al. (2008) for *T. equi* and Bhoora et al. (2010), for *B. caballi*. These methods were subsequently chosen following a study carried out for the evaluation of the sensitivity and specificity of some of the PCR methods reported in literature or commercially available (Antonella Cersini, unpublished results).

2.3.1. DNA extraction

DNA blood extraction was performed using the automated robotic workstation QIAcube HT (Qiagen, GmbH, Hilden, Germany) and the QIAamp cador Pathogen Mini kit (Qiagen) according to the manufacturers instructions. The DNA was eluted in 150 μl AVE buffer included in the kit, composed of RNAase-free water containing 0.04% NaN_3 and stored at −80 °C.

2.3.2. Real time PCR for B. caballi and T. equi

RtPCR for *T. equi* amplified an 81 bp fragment outside the V4 hypervariable region of the 18S rRNA gene. Primers (F:Be18SF; R:Be18SR) and TaqMan probe (VIC-TAMRA, Be 18SP) were those reported by Kim et al. (2008). RtPCR for *B. caballi* amplified a 95 bp fragment in the V4 hypervariable region of the 18S rRNA gene of *B. caballi*. Primers (F: Bc-18SF402; R: Bc-18SR496) and TaqMan MGB™ probe (FAM-MGB, Bc-18SP) were those reported by Bhoora et al. (2010). For both rtPCRs, TaqMan® Universal PCR Master Mix kit (A. Biosystems, Foster City, CA, USA) was used. Internal positive controls were rtPCR products of *B. caballi* and *T. equi*, obtained from EDTA blood samples of seropositive symptomatic subjects, certified by the Office International des Epizooties (OIE) Reference Laboratory for Babesiosis of the Istituto Zooprofilattico Sperimentale della Sicilia, and cloned in the plasmid vector PCRII®-TOPO® (Invitrogen, Carlsbad, CA, USA). The negative control used in the reactions was RNAase-free water.

The rtPCRs were carried out using ABIPRISM 7900 HT Sequence Detection System (A. Biosystems).

2.3.3. Sequencing

The specificity of the rtPCR results was verified by sequencing the amplicons of some of the PCR positive samples (44) obtained using a nested PCR protocol as described by Nagore et al. (2004), amplifying the hypervariable V4 region of the 18 rRNA gene of both protozoa. The amplicons were sequenced using an automated sequencer (3500 Genetic Analyzer, A. Biosystems, Foster City, CA, USA) and the nucleotide sequences obtained were analysed using the Genetic Analyzer Sequencing v5.4 (A. Biosystems, Foster City, CA, USA). Sequence identity was verified using the Basic Local Alignment Search Tool (BLAST) and by comparing those obtained with *B. caballi* and *T. equi* sequences present in NCBI GenBank (http://www.ncbi.nlm.nih.gov). Sequences presenting an identity and query coverage ≥ 98% were considered as homologous to those deposited in GenBank for the two piroplasms (Marasca et al., 2005).

2.4. Statistical analysis

Serological and PCR-positivity prevalence with a 95% CI were calculated as described by Thrusfield (2007), for the study area, for the province in which the horses resided and for each risk factor investigated. The association between explanatory variables and positivity for each piroplasm was verified in two stages. In a first step, associations between serological and PCR-positivity prevalence for *T. equi* and *B. caballi* and for each risk factor considered were assessed using Chi square or Fisher's exact test. Those resulting significant (p value ≤ 0.05, two-tailed) were then included in a stepwise backward logistic regression. STATA SE v.12.0 software for Windows (StataCorp LP, Texas, USA) was used for all statistical analyses.

3. Results

The study was conducted on 673 long-term resident horses in an area across the Regions of Latium, Molise and Campania of Central-Southern Italy. Serological and PCR-positivity prevalence levels for both parasites and each province are shown in Fig. 1. Of the total 273 seropositive samples, five were excluded from the analysis of the PCR-positive outcome as they contained inhibiting factors. The province with highest seroprevalence and PCR-positivity level for both parasites was Frosinone.

3.1. T. equi

3.1.1. T. equi seroprevalence

Seroprevalence values, relative 95% CIs and p-values of the statistical tests obtained for each variable included in the risk analysis are shown in Table 1.

The overall seroprevalence for *T. equi* was 39.8% (95% CI: 36.0–44.0%), ranging from 25.0% (Salerno) to 64.6% (Frosinone).

In a preliminary evaluation in the univariate model for gender including three categories (gelding, male and female), no differences were observed between the first two groups that were therefore unified in a single category for subsequent analysis. The variables resulting significant ($p \leq 0.05$) in the univariate model were gender, age, breed, access to pasture, altitude, land cover, soil type and province location. In particular, for gender and age,

Table 1
Results of the univariate analyses for *T. equi* and *B. caballi* seroprevalence. *P* value ≤ 0.05 was considered significant.

			T. equi			*B. caballi*		
Variables	Category	*N*	Prevalence (%)	95% CI	*p*	Prevalence (%)	95% CI	*p*
Gender								
	Male	344	29.9	(25.3–35.0)	<0.001	2.9	(1.6–5.3)	<0.001
	Female	329	50.2	(44.8–55.5)		15.2	(11.7–19.5)	
Age (years)								
	≤6	214	31.8	(25.9–38.3)	<0.01	10.7	(7.3–15.6)	0.08
	7–12	237	43.9	(37.7–50.2)		11.0	(7.6–15.6)	
	>12	204	44.6	(37.9–51.5)		5.4	(3.0–9.4)	
Breed								
	Foreign	203	30.5	(24.6–37.2)	<0.01	2.5	(1.1–5.6)	<0.001
	Italian	260	41.5	(35.7–47.6)		9.6	(6.6–13.8)	
	Mixed	210	46.7	(40.0–53.4)		14.3	(10.2–19.7)	
Access to pasture								
	No	192	31.3	(25.1–38.1)	<0.01	1.6	(0.5–4.5)	<0.001
	Yes	481	43.2	(38.9–47.7)		11.9	(9.3–15.0)	
Altitude (m)								
	<150	328	34.1	(29.2–39.4)	<0.01	1.2	(0.5–3.1)	<0.001
	150–600	275	47.3	(41.5–53.2)		20.0	(15.7–25.1)	
	>600	70	37.1	(26.8–48.9)		1.4	(0.3–7.7)	
Land cover								
	>75% forest	65	44.6	(33.2–56.7)	<0.01	3.1	(0.8–10.5)	<0.001
	50–75% crops	184	40.2	(33.4–47.4)		8.7	(5.4–13.7)	
	50–75% forest	116	26.7	(19.5–35.4)		0.9	(0.2–4.7)	
	Mixed	308	43.5	(38.1–49.1)		13.3	(10.0–17.6)	
Climatic zone								
	Humid	393	37.2	(32.5–42.0)	0.114	3.3	(1.9–5.6)	<0.001
	Sub-Humid	260	42.3	(36.5–48.4)		17.7	(13.5-22.8)	
	Moist-semiarid	20	60.0	(38.7–78.1)		5.0	(0.9–23.6)	
Soil type								
	Eutric Cambisol	134	44.0	(35.9–52.5)	<0.001	2.2	(0.8–6.4)	<0.001
	Dystrict cambisol	216	31.5	(25.7–38.0)		1.4	(0.5–4.0)	
	Andosol	177	25.4	(19.6–32.3)		0	(0–2.1)	
	Chromic Luvisol	146	65.8	(57.7–73.0)		37.0	(29.6–45.1)	
Province location								
	Coastal	332	31.3	(26.6–36.5)	<0.001	4.2	(2.5–7.0)	<0.05
	Inland	341	48.1	(42.8–53.4)		13.5	(10.3–17.5)	

N = number of samples tested; 95% CI = confidence interval; *p* = *p* value

prevalence was higher in females ($p < 0.001$) and increased with age ($p < 0.01$). Relative to the breed categories, seroprevalence in the mixed breed was significantly higher ($p < 0.01$) than in the other two groups, followed by the Italian breed. In horses with access to pasture prevalence was significant with $p < 0.01$. For altitude, the highest prevalence was found in the group resident at 150–600 m asl ($p < 0.01$), while prevalence was higher in the inland provinces than in the coastal ones ($p < 0.001$).

The final multivariate model included seven risk factors represented by gender, age, breed, access to pasture, land cover, soil type and province location (Table 2).

3.1.2. T. equi *PCR-positivity prevalence*

Values of the PCR-positivity prevalence, relative 95% CIs and p-values of the statistical tests obtained for each variable included in the risk analysis, are shown in Table 3.

T. equi PCR-positivity was detected in 70.3% of samples (95% CI: 64.6–75.5%), ranging from 38.5% (Salerno) to 81.4% (Frosinone). In the univariate analysis, gender, age, breed, access to pasture, altitude, land cover, climatic zone, soil type and province location were the variables found significant ($p \leq 0.05$).

For this outcome, prevalence level order within the significant variables was similar to that described for seropositivity with the exception of age and altitude. In fact, PCR-positivity prevalence significantly decreases with age ($p < 0.001$), while increases with altitude ($p < 0.05$).

The risk factors identified for this outcome in the multivariate model were age, soil type and province location (Table 4).

Table 2
Results of multivariate logistic analysis of risk factors for *T. equi* seroprevalence. *P* value ≤ 0.05 was considered significant.

Variables	Category	*p*	Odds ratio	95% CI
Gender				
	Male	a		
	Female	0.001	1.86	(1.27–2.71)
Age (years)				
	≤6	a		
	7–12	0.002	2.09	(1.32–3.31)
	>12	<0.0001	2.59	(1.61–4.16)
Breed				
	Foreign	a		
	Italian	<0.0001	3.57	(1.99–6.41)
	Mixed	0.002	2.51	(1.38–4.55)
Access to pasture				
	No	a		
	Yes	0.004	2.22	(1.29–3.83)
Land cover				
	>75% forest	a		
	50–75% crops	0.170	0.56	(0.25–1.27)
	50–75% forest	0.020	0.42	(0.20–0.87)
	Mixed	0.564	0.76	(0.29–1.95)
Soil type				
	Eutric Cambisol	a		
	Dystrict cambisol	0.001	0.29	(0.14–0.60)
	Andosol	0.084	0.49	(0.21–1.10)
	Chromic Luvisol	0.002	3.16	(1.54–6.47)
Province location				
	Coastal	a		
	Inland	0.005	2.5	(1.33–4.74)

N = number of samples tested; 95% CI = confidence interval; *p* = value; *a* = baseline

Table 3
Results of univariate analyses for *T. equi* and *B. caballi* PCR-positive prevalence. *P* value ≤ 0.05 was considered significant.

			T. equi			*B. caballi*		
Variables	Category	*N*	Prevalence (%)	95% CI	*p*	Prevalence (%)	95% CI	*p*
Gender								
	Male	100	61.0	(51.2–70.0)	<0.01	8.0	(4.1–15.0)	0.34
	Female	163	76.1	(69.0–82.0)		11.7	(7.6–17.5)	
Age (years)								
	≤6	67	83.6	(72.9–90.6)	<0.001	14.9	(8.3–25.3)	<0.05
	7–12	104	74.0	(64.9–81.5)		13.5	(8.2–21.3)	
	>12	81	61.7	(50.8–71.6)		3.4	(1.2–9.7)	
Breed								
	Foreign	61	57.4	(44.9–69.0)	<0.01	11.5	(5.7–21.8)	0.90
	Italian	104	69.2	(59.8–77.3)		10.6	(6.0–18.0)	
	Mixed	98	79.6	(70.6–86.4)		9.2	(4.9–16.5)	
Access to pasture								
	No	57	57.9	(41.1–73.0)	<0.05	5.3	(1.8–14.4)	0.22
	Yes	206	73.8	(65.2–80.8)		11.7	(8.0–16.7)	
Altitude (m)								
	<150	107	62.6	(53.2–71.2)	<0.05	5.6	(2.6–11.7)	0.07
	150–600	129	72.9	(64.6–79.8)		14.7	(9.6–21.9)	
	>600	27	88.9	(71.9–96.1)		7.4	(2.1–23.4)	
Land cover								
	>75% forest	30	86.7	(70.3–94.7)	<0.05	10.0	(3.5–25.6)	0.60
	50–75% crops	74	78.4	(67.7–86.2)		10.8	(5.6–19.9)	
	50–75% forest	32	59.4	(42.3–74.5)		3.1	(0.6–15.7)	
	Mixed	127	64.6	(55.9–72.3)		11.8	(7.3–18.6)	
Climatic zone								
	Humid	141	60.3	(52.0–68.0)	<0.001	5.7	(2.9–10.8)	<0.05
	Sub-Humid	109	81.7	(73.4–87.8)		16.5	(10.7–24.6)	
	Moist-semiarid	13	84.6	(57.8–95.7)		7.7	(1.4–33.3)	
Soil type								
	Eutric Cambisol	59	64.4	(51.7–75.4)	<0.001	13.6	(7.0–24.5)	<0.05
	Dystrict cambisol	70	77.1	(66.0–85.4)		4.3	(1.5–11.9)	
	Andosol	44	43.2	(29.7–57.8)		2.3	(0.4–11.8)	
	Chromic Luvisol	90	82.2	(73.1–88.8)		16.7	(10.4–25.7)	
Province location								
	Coastal	102	55.9	(46.2–65.1)	<0.001	4.9	(2.1–11.0)	<0.05
	Inland	161	79.5	(72.6–85.0)		13.7	(9.2–19.8)	

N = number of samples tested; 95% CI = confidence interval; *p* = *p* value.

Table 4
Results of multivariate logistic analysis of risk factors for *T. equi* and *B. caballi* PCR-positive prevalence. *P* value ≤ 0.05 was considered significant.

T. equi

Variables	Category	*p*	Odds Ratio	95% CI
Age (years)				
	≤6	a		
	7–12	0.110	0.51	(0.22–1.16)
	>12	0.004	0.29	(0.12–0.66)
Soil				
	Eutric Cambisol	a		
	Dystrict Cambisol	0.04	3.59	(1.49–8.66)
	Andosol	0.354	1.71	(0.55–5.29)
	Chromic Luvisol	0.001	4.55	(1.87–1.08)
Province location				
	Coastal	a		
	Inland	0.007	2.91	(1.33–6.35)

B. caballi

Variables	Category	p	Odds Ratio	95% CI
Climatic zone				
	Humid	a		
	Sub-Humid	0.009	3.55	(1.38–9.16)
	Moist-semiarid	0.185	5.83	(0.43–9.17)
Soil				
	Eutric Cambisol	a		
	Dystrict Cambisol	0.020	0.14	(0.03–0.74)
	Andosol	0.091	0.16	(0.02–1.34)
	Chromic Luvisol	0.749	0.85	(0.31–2.30)

N = number of samples tested; 95% CI = confidence interval; *p* = value; *a* = baseline.

3.2. B. caballi

3.2.1. B. caballi *seroprevalence*

The overall seroprevalence was 8.9% (95% CI: 7.0–11.3%), ranging from 0% (Naples, Isernia and Caserta) to 26.8% (Frosinone). The following variables were significant ($p \leq 0.05$) in the univariate analysis: gender, breed, access to pasture, altitude, land cover, climatic zone, soil type and province location (Table 1).

Females had a significant higher seroprevalence than males ($p < 0.001$). Significant differences ($p < 0.001$) due to the breed and access to pasture were similar to those described for *T. equi* (Table 1).

The multivariate model was not performed for *B. caballi* seroprevalence due to the low number or absence of positive animals for the different variables.

3.2.2. B. caballi *PCR positivity prevalence*

PCR-positivity for this parasite was present in 10.3% of the samples examined (95% CI: 7.2–14.5%), ranging from 0% (Naples and Salerno) to 17.6% (Frosinone) (Table 3). The following variables resulted significant ($p \leq 0.05$) in the univariate model: age, climatic zone, soil type and province location with prevalence decreasing significantly with age ($p < 0.05$) as shown in Table 3.

Climatic zone and soil type were the risk factors found to be associated in the multivariate model with *B. caballi* seropositivity (Table 4).

3.3. Sequencing

All the 44 PCR-positive samples had a sequence identity of ≥98% with the equine piroplasms deposited in GenBank. A detailed phylogenetic analysis study will be described in another paper.

4. Discussion

In this study, 673 samples were initially examined from asymptomatic horses to determine the seroprevalence for each of the equine piroplasms. Subsequently, 273 seropositive samples were tested in PCR for each parasite that could better correlate with a carrier status. These outcomes were then used to identify associated risk factors.

Seroprevalence for *T. equi* was 39.8%, in line with those of other studies carried out in Italy using IFAT, where the prevalence values reported were 41.0% (Laus et al., 2013) and 50.5% (Moretti et al., 2010), and in some Mediterranean countries, with 33.7% in Israel (Shkap et al., 1998) using an in-house cELISA and 50.3% in Spain (García-Bocanegra et al., 2013) using cELISA VMRD®. In our study, the results obtained indicate that the factors influencing prevalence are apparently homogeneous throughout the geographic area of interest, even if quite wide in extension.

T. equi PCR-positivity prevalence was 70.3%, even if analogous studies conducted in Italy described lower percentages (Grandi et al., 2011, Moretti et al., 2010) which could be attributed to the different PCRs employed (End point vs Real time) and target choice (type and length), that are factors influencing the sensitivity of the method.

Seroprevalence for *B. caballi* was 8.9%, in accordance with the results of a Spanish study (8.4%) (García-Bocanegra et al., 2013) but higher than those of other results that were around 2%, as obtained in Greece (Kouam et al., 2010) and in Turkey (Sevinc et al., 2008). In our case, the higher seroprevalence could be due to an infection cluster on the Aurunci Mountains, discussed in more detail below. On the other hand, Italian studies using IFAT described by Laus et al. (2013) and Moretti et al. (2010) respectively reported markedly higher prevalence values, 26% and 56%, that could be related either to the location of their study areas, as both authors sampled horses from other Italian regions or to the assay's characteristics.

PCR-positivity prevalence for *B. caballi* was 10.3%, which is higher than in other studies performed in the same country (Grandi et al., 2011, Moretti et al., 2010). The considerations proposed for the *T. equi* PCR-positivity prevalence could also be valid for *B. caballi*, i.e. due to the infection cluster previously mentioned that was also found for this parasite.

This infection cluster was located on the Aurunci Mountains, a Regional Park, location of which is shown in Fig. 1, ranging from 30 to 1535 m asl, hosting a wide variety of flora (beech, oak, apple, chestnut, maple trees) and fauna, including equine piroplasm hosts and vectors. Ninety-four samples were collected from horses within this area, and for *B. caballi*, 43 (45.7%) were seropositive and 16 (37%) were PCR-positive, while for *T. equi*, 75 (79.8%) were seropositive and 66 (88%) were PCR-positive. This cluster of positivity could be ascribed to the characteristics of the territory particularly favourable in maintaining a high number of asymptomatic infections caused by equine piroplasms, probably due to absence of management and a particular adaptability of the horses present in this area to these parasites.

Gender was a risk factor common to both parasites, with females showing a higher positivity than males. As already proposed in another study (Rüegg et al., 2007), the difference observed by us could be due to gender-specific management practices. In the multivariate model, females showed an odds ratio (OR) of 1.86 for *T. equi* seroprevalence. Similar results were observed by Moretti et al. (2010) but were not discussed.

Differences related to age were significant in the univariate analysis for *B. caballi* PCR-positivity and in the multivariate model for *T. equi* (seroprevalence and PCR-positivity). For *B. caballi*, PCR-positivity decreases with age that could be attributed to a parasite clearance occurring in around 4 years, with the subsequent disappearance of antibodies (De Waal, 1992). This data appears to be in line to what has been found in a previous study (Rüegg et al., 2007), in which, in addition to parasite clearance, exclusive localization of *B. caballi* at the microvasculature level is hypothesized to explain the age-dependant decrease in PCR-positivity. An alternative hypothesis could be that older horses become more efficient in eliminating or maintaining the parasitic load lower than the PCR detection limit, depending on a cell-mediated immunity mechanism. Moreover, according to Rüegg et al. (2008) when a horse clears a *B. caballi* infection, the mean time of re-infection is in the order of 14 years, unlikely to occur in a species with a life expectancy of around 20 years.

Differently, *T. equi* seroprevalence increases with age, with the older group showing an OR of 2.59, respect to the baseline, related to a chronic infectious status (De Waal, 1992) causing a cumulative positivity. These observations are in agreement with other authors (Kouam et al., 2010; García-Bocanegra et al., 2013). However, for *T. equi* PCR-positivity, OR decreases with age, in contrast with a previous study carried out in Mongolia, that highlighted a cumulative age-dependent increase of this result (Rüegg et al., 2008) and with the study of Steinman (2012) in Israel, that reported no significant differences within the age classes considered. The circumstances that influence the presence of *T. equi* in the blood, across the life of a horse, require further verification if due to the sensitivity of the various methods used in the different studies or to host–parasite interactions. Significant differences associated with horse breeds were found in the univariate analyses for *B. caballi* seropositivity and *T. equi* PCR-positivity, while in the multivariate analysis only the latter was significant. Italian breed and mixed breed horses respectively had an OR of 3.57 and 2.51 of being T. *equi* seropositive than foreign breed horses. These dissimilarities could be related to various management practices, in that rearing conditions of foreign pure breeds lead to a lower exposure to EP, even if other authors hypothesised that differences in susceptibility could be breed dependent (Steinman et al., 2012, Sevinc et al., 2008). As all horses included in the study were healthy and asymptomatic, even if some could have been carriers, this indicates a grade of parasitic tolerance and disease resistance. Mixed breeds are usually more robust while local breeds are more adapted to their environment. In a study conducted on the major histocompatibility complex genetic diversity in donkeys, related to EP resistance, the authors report that this is associated with the effects of breeding and different genetic origins of the studied populations rather than pathogen-driven selection (Vranova et al., 2011). A similar study in horses would assist in clarifying if our results are comparable with the findings of Vranova et al. (2011).

Access to pasture was a significant variable in the univariate model for *B. caballi* and *T. equi* seropositivity and *T. equi* PCR-positivity while in the multivariate analysis it was only significant for *T. equi* seroprevalence. Similarly to what was observed by other authors (Kouam et al., 2010; García-Bocanegra et al., 2013; Shkap et al., 1998, Moretti et al., 2010, Steinman et al., 2012), horses kept on pastures were 2.22 times more likely to be seropositive to *T. equi*, presumably due to a greater tick-exposure or to lack of grooming that aids tick removal.

Altitude was significant only in the univariate model for the seroprevalence of both piroplasms and for *T. equi* PCR-positivity. Highest number of seropositives were found for both parasites at 150–600 m asl, while for *T. equi* PCR-positivity this condition was

at found at the > 600 m asl category. In our case, the difference in altitude and outcome could be associated with a seasonal effect on the presence of ticks and prevalence of PCR-positivity. Even for this, as no data is available, ticks are being collected in the area to study their distribution and positivity for the equine piroplasms. Movement of the horses between the different altitude categories could also influence the significance of this variable on the outcomes, however considered irrelevant as the study animals were confined to the premises where they were living.

Land cover was significant in the univariate analyses for *B. caballi* seroprevalence and *T. equi* PCR-positivity, with a respectively higher prevalence in animals living in mixed zones (with no dominance of a particular land cover category) and in areas with more than 75% forest coverage. In the multivariate model for *T. equi* seropositivity, land cover was again a significant variable, with an OR of 0.42 in the 50–75% forest compared to the >75% forest category. These results are in line with those described by Vanwambeke et al. (2010) in which the author reported that arable fields, or patches of forests surrounded by agricultural lands, have a favourable impact on the control of vector-borne diseases, contrary to those with a high percentage of forest land, most likely because of a less suitable environment for the presence of ticks in the former type of land cover, related to its agricultural use.

The climatic zone in the univariate analysis was significant for *B. caballi* seroprevalence and PCR-positivity and for *T. equi* PCR-positivity while in the multivariate analysis it was only significant for *B. caballi* PCR-positivity. For both *B. caballi* outcomes, sub-humid zones (LGP 180-269 days) revealed higher prevalence levels and a PCR-positivity OR of 3.55. Moist semiarid zones (LGP 120-179) also had a higher prevalence for *T. equi* PCR-positivity in the univariate analysis. In our case, a greater number of samples could aid in confirming these results.

Soil type was a significant variable for both equine piroplasms for the outcomes analysed in the univariate and multivariate models. In the univariate analysis, for both parasites and outcomes, the chromic luvisol group showed the highest number of sero/PCR-positive animals. In the *T. equi* multivariate models, subjects belonging to the chromic luvisol group presented an OR of 3.16 (seropositivity) and 4.55 (PCR-positivity) while those of the dystric group showed a significant OR of 3.58, only for the *T. equi* PCR-positive outcome. On the contrary, in the multivariate model, for the *B. caballi* PCR-positive outcome, the dystric group showed an OR < 1 compared to the baseline and no differences were detected between the chromic luvisol and the eutric group. This result indicates the importance of the influence of soil type on the interaction between host, parasite and environment. To our knowledge there are no reports that include soil type with this classification in studies similar to ours, however Schwarz et al. (2009) discuss the indirect influence of soil type on vegetation and distribution of ticks. While land cover and land use should be first choice parameters, in the absence of specific information, a correlated variable that is soil type was included. As mentioned earlier, a study is currently ongoing in the same study area to investigate the vector's ecology related to the soil type.

The inland provinces showed an OR respectively of 2.5 for seroprevalence and 2.9 for PCR-positivity for *T. equi* with respect to the coastal provinces that could be attributed to a more suitable vector habitat, as these provinces tend to have a higher altitude and greater forest coverage with respect to the former.

Our data indicates that the levels of seroprevalence and PCR-positivity for EP are influenced by abiotic and biotic characteristics and their interactions, which determine the tick population and consequently the level of exposure to the pathogens. Climate, microclimate, humidity, soil temperature and pore size, altitude, urbanization and adaptation of ticks to new environments and finally presence of the host have been described by Pfäffle et al. (2013) as related to tick distribution, abundance and behaviour. All these factors are essential for the constant maintenance of vectors and parasites (Scoles and Ueti, 2015).

Although some of the variables considered in the present study were significant in the univariate analysis but not in the multivariate, it is important that they are still taken into consideration as they might have a different behaviour under other study conditions or when including other or different risk factors in the analysis. A confirmation of this is that for some of the risk factors evaluated, other authors reported different statistical outcomes (Steinman et al., 2012). Among the risk factors that should be investigated, it would be highly important to also include tick species present in the study area and related parameters, such as land cover and land use, even if information on these parameters is rarely present.

5. Conclusion

This survey defines the presence of a high seroprevalence as well as a high number of asympomatic PCR-positive horses for both parasites, with a cluster located within the Latium region. Several risk factors associated with the host and the environment were significantly related to EP positivity, confirming the observations of previous studies in the Mediterranean area. However, further investigations on the influence of environmental factors are required and particularly on tick ecology and distribution in this area. Although pharmacological treatment and prophylaxis for EP are available, the side effects of these interventions can lead to serious complications such as hepatotoxicity and nephrotoxicity (Donnellan and Marais, 2009). On the basis of the results obtained in this study, control programmes could be developed, specifically based on known local risk factors with the adoption of suitable practices including tick control, land usage and suitable horse management. Such preventive measures would aid in limiting pharmacological interventions, advantageous for the host as well as for reducing the likelihood of establishing parasitic resistance.

6. Conflict of interest

The authors declare no conflict of interests.

Acknowledgements

This study was conducted within the IZSLT 16/11, research project funded by the Italian Ministry of Health.

The authors wish to thank Dr. Claudio De Liberato and Dr. Adele Magliano for their entomological support and Dr. Sabrina Battisti for her assistance for GIS.

References

Bhoora, R., Quan, M., Franssen, L., Butler, C.M., Van der Kolk, J.H., Guthrie, A.J., Zweygarth, E., Jongejan, F., Collins, N.E., 2010. Development and evaluation of real-time PCR assays for the quantitative detection of Babesia caballi and Theileria equi infections in horses from South Africa. Vet. Parasitol. 168, 201–211, http://dx.doi.org/10.1016/j.vetpar.2009.11.011.

De Waal, D.T., 1992. Equine piroplasmosis: a review. Br. Vet. J. 148, 6–14, http://dx.doi.org/10.1016/0007-1935(92)90061-5.

Donnellan, C.M.B., Marais H.J., 2009. Equine piroplasmosis. In Infectious Diseases of the Horse, ed. Mair TS, Hutchinson RE. E V J; 333-40.

Friedhoff, K.T., Tenter, A.M., Müller, I., 1990. Haemoparasites of equines: impact on international trade of horses. Rev. Sci. Tech. Off. Int. Epiz. 9 (4), 1187–1194.

García-Bocanegra, I., Arenas-Montes, A., Hernández, E., Adaszek, Ł., Carbonero, A., Almería, S., Jaén-Téllez, J.A., Gutiérrez-Palomino, P., Arenas, A., 2013. Seroprevalence and risk factors associated with Babesia caballi and Theileria equi infection in equids. Vet. J. 195, 172–178, http://dx.doi.org/10.1016/j.tvjl.2012.06.012.

Grandi, G., Molinari, G., Tittarelli, M., Sassera, D., Kramer, L.H., 2011. Prevalence of Theileria equi and Babesia caballi infection in horses from northern Italy.

Vector Borne Zoonotic Dis. 11, 955–956, http://dx.doi.org/10.1089/vbz.2010.0193.
Kim, C.M., Blanco, L.B.C., Alhassan, A., Iseki, H., Yokoyama, N., Xuan, X., Igarashi, I., 2008. Diagnostic real-time PCR assay for the quantitative detection of Theileria equi from equine blood samples. Vet. Parasitol. 151, 158–163, http://dx.doi.org/10.1016/j.vetpar.2007.10.023.
Kouam, M.K., Kantzoura, V., Masuoka, P.M., Gajadhar, A. a., Theodoropoulos, G., 2010. Genetic diversity of equine piroplasms in Greece with a note on speciation within Theileria genotypes (T. equi and T. equi-like). Infect. Genet. Evol. 10, 963–968, http://dx.doi.org/10.1016/j.meegid.2010.06.008.
Laus, F., Veronesi, F., Passamonti, F., Paggi, E., Cerquetella, M., Hyatt, D., Tesei, B., Fioretti, D.P., 2013. Prevalence of tick borne pathogens in horses from Italy. J. Vet. Med. Sci. 75, 715–720, http://dx.doi.org/10.1292/jvms.12-0449.
Marasca, R., Maffei, R., Morselli, M., Zucchini, P., Castelli, I., Martinelli, S., Torelli, G., 2005. Immunoglobulin mutational status detected through single-round amplification of partial V(H) region represents a good prognostic marker for clinical outcome in chronic lymphocytic leukemia. J. Mol. Diagn. 7 (5), 566–574, http://dx.doi.org/10.1016/S1525-1578(10)60589-2.
Moretti, A., Mangili, V., Salvatori, R., Maresca, C., Scoccia, E., Torina, A., Moretta, I., Gabrielli, S., Tampieri, M.P., Pietrobelli, M., 2010. Prevalence and diagnosis of Babesia and Theileria infections in horses in Italy: A preliminary study. Vet. J. 184, 346–350, http://dx.doi.org/10.1016/j.tvjl.2009.03.021.
Nagore, D., García-Sanmartín, J., García-Pérez, A.L., Juste, R.a., Hurtado, A., 2004. Detection and identification of equine Theileria and Babesia species by reverse line blotting: Epidemiological survey and phylogenetic analysis. Vet. Parasitol. 123, 41–54, http://dx.doi.org/10.1016/j.vetpar.2004.04.010.
Pfäffle, M., Littwin, N., Muders, S.V., Petney, T.N., 2013. The ecology of tick-borne diseases. Int. J. Parasitol. 43, 1059–1077, http://dx.doi.org/10.1016/j.ijpara.2013.06.009.
Rüegg, S.R., Torgerson, P., Deplazes, P., Mathis, A., 2007. Age-dependent dynamics of Theileria equi and Babesia caballi infections in southwest Mongolia based on IFAT and/or PCR prevalence data from domestic horses and ticks. Parasitology 134, 939–947, http://dx.doi.org/10.1017/S0031182007002405.
Rüegg, S.R., Heinzmann, D., Barbour, A.D., Torgerson, P.R., 2008. Estimation of the transmission dynamics of Theileria equi and Babesia caballi in horses. Parasitology 135 (5), 555–565, http://dx.doi.org/10.1017/S0031182008004204.
Scoles, G., Ueti, M.W., 2015. Vector ecology of equine piroplasmosis. Annu. Rev. Entomol. 60, 561–580, http://dx.doi.org/10.1146/annurev-ento-010814-021110.
Sevinc, F., Maden, M., Kumas, C., Sevinc, M., Ekici, O.D., 2008. A comparative study on the prevalence of Theileria equi and Babesia caballi infections in horse sub-populations in Turkey. Vet. Parasitol. 156, 173–177, http://dx.doi.org/10.1016/j.vetpar.2008.06.006.
Shkap, V., Cohen, I., Leibovitz, B., Savitsky, Pipano, E., Avni, G., Shofer, S., Giger, U., Kappmeyer, L., Knowles, D., 1998. Seroprevalence of Babesia equi among horses in Israel using competitive inhibition ELISA and IFA assays. Vet. Parasitol. 76, 251–259.
Schwarz, A., Maier, W.a., Kistemann, T., Kampen, H., 2009. Analysis of the distribution of the tick Ixodes ricinus L. (Acari: Ixodidae) in a nature reserve of western Germany using Geographic Information Systems. Int. J. Hyg. Environ. Health 212, 87–96, http://dx.doi.org/10.1016/j.ijheh.2007.12.001.
Steinman, A., Zimmerman, T., Klement, E., Lensky, I.M., Berlin, D., Gottlieb, Y., Baneth, G., 2012. Demographic and environmental risk factors for infection by Theileria equi in 590 horses in Israel. Vet. Parasitol. 187, 558–562, http://dx.doi.org/10.1016/j.vetpar.2012.01.018.
Sumbria, D., Das Singla, L., Veterinary, D., Scien, A., Das, L., Guru, S., Dev, A., Scien, A., 2015. Recent Diagnostic and control approaches in Equine piroplasmosis.
Thrusfield, M., 2007. Veterinary Epidemiology, 3rd Edition. Wiley-Blackwell, USA.
Vanwambeke, S.O., Sumilo, D., Bormane, A., Lambin, E.F., Randolph, S.E., 2010. Landscape predictors of tick-borne encephalitis in Latvia: land cover, land use, and land ownership. Vector Borne Zoonotic Dis. 10, 497–506, http://dx.doi.org/10.1089/vbz.2009.0116.
Vranova, M., Alloggio, I., Qablan, M., Vyskocil, M., Baumeisterova, A., Sloboda, M., Putnova, L., Vrtkova, I., Modry, D., Horin, P., 2011. Genetic diversity of the class II major histocompatibility DRA locus in European Asiatic and African domestic donkeys. Infect. Genet. Evol. 11, 1136–1141, http://dx.doi.org/10.1016/j.meegid.2011.04.010.

Web references

1. http://www.fao.org/geonetwork/srv/en/main.home, 1/09/2015.
2. http://www.fao.org/ag/againfo/programmes/en/lead/toolbox/Refer/AgroeZon.htm, 1/09/2015.
3. http://www.isric.org/sites/all/modules/pubdlcnt/pubdlcnt.php?file=http://library.wur.nl/isric/fulltext/isricu_i9264_001.pdf&nid=333, 1/09/2015.
4. http://www.fao.org/docrep/019/as353e/as353e.pdf, 1/09/2015.

horses is the Complement Fixation Test (CFT). However, studies have shown that CFT cannot differentiate between species of Trypanosoma as the CFT is not species specific [5]. The diagnostic significance of this test is therefore doubtful in countries where both *T. equiperdum* and *T. evansi* infections occur. Human sleeping sickness [6] caused by *T. gambiense* and surra in camels caused by *T. evansi* [2] can be diagnosed with *T. equiperdum* antigen. In collaboration with the Instituto G. Caporale, Teramo, Italy, we received 8 confirmed Dourine positive equine sera samples of different titres collected during the Italian outbreak in 2011 [4]. These 8 sera were further tested at CVRL using antigen preparations from 7 different *T. equiperdum* strains including ITMAS 170108 (OVI), ITMAS 070109 (Botat), ITMAS 261003B 943 (Ethiopian), ITMAS 220101 (American), ITMAS 211199A (French), ITMAS 241199B (German), ITMAS 290101 (Canadian), as well as *T. evansi* strain. *T. evansi* strain was isolated from a dromedary in Dubai [3]. The results of these investigations are summarized in Table 1. As seen from the Table, 8 sera reacted with different titres against different *T. equiperdum* strains as well as *T. evansi.* This proves that standardization of *T. equiperdum* antigen for use in CFT is an important issue.

Table 1
CFT results of 8 Dourine-positive Italian horse sera using *T. evansi* and different strains of *T. equiperdum* antigens

SAMPLE ID	CVRL CFT	CFT	CVRL CFT ** (with different *T. equiperdum* strains)						
	*T. evansi**	Teramo, Italy	Ethiopian Ag	Ovi Ag	French Ag	Canadian Ag	German Ag	BoTat Ag	American Ag
NAM 3	1:20++++	1:320++	1:160+	1:80++	1:40++	1:40+++	1:80++++	1:80+	1:80+
NAM 4	1:40++	1:640++	1:160++	1:80++	1:40++	1:80+	1:160+	1:80+	1:80+++
BATCH 0036	1:20+++	1:2560	1:40+	1:40+++	1:40+	1:40+	1:80+	1:20++	1:80++
BATCH 0037	1:20+	1:160	1:40++	1:40+++	1:20+	1:40+	1:80+	1:40+	1:40++
BATCH 0039	Negative	1:10	1:2++	1:5++	1:2+	1:2+	1:5++	1:2++	1:5++
BATCH 0038	1:160+	1:2560	1:640++	1:320++	1:320+	1:320+	1:320+	1:320+	1:640++
BATCH 0040	1:10++++	1:160	1:40+	1:40+	1:20+	1:10+++	1:80+	1:20++	1:40++
BATCH 0041	1:5++++	1:160	1:10+	1:5+++	1:2+++	1:5+++	1:10++	1:10+	1:5+++

*The optimum dilution of *T. evansi* antigen for CFT testing has been obtained by performing checker board with *T. evansi* positive serum

**The optimum dilution of different isolates of *T. equiperdum* antigen for CFT testing has been obtained by performing checker board with *T. equiperdum* positive serum.

References

[1] Gallo C., Vesco G., Campo F., Haddad N. & Awelmoula H. (1989). — Enquête zoo-sanitaire chez les chèvres et les dromadaires au sud de la Tunisie. Maghreb Vét., 4, 15-17.

[2] Kinne J., Wernery U. & Zachariah R. (2001). Surra in a guanaco (*Lama guanicoe*). JCPR, 8(2), 93-98.

[3] Pascucci I. & Cammà C. (2015). Updates and lessons from the 2011 outbreak in Italy, Workshop of the EU RL on Dourine, 2 October 2015, Paris-France.

[4] Pautrizel R., Lafaye A. & Duret J. (1959). — Diagnostic sérologique de la maladie du sommeil. 1. Amélioration de l'antigène préparé à partir de T. equiperdum. Bull. Soc. Pathol. Exot., 52, 318-331.

[5] Pautrizel R., Lafaye A., Duret J., Tribouley J. & Ripert C. (1962). — Etude comparative de l'évolution de différents anticorps élaborés au cours de trypanosomose expérimentale chez le lapin. Rev. Immunol. Fr., 26, 157-166.

187
Preliminary investigations on the sequence heterogeneity of the 18S rRNA gene of *Theileria equi* and *Babesia caballi* strains collected from a horse population in Central Italy

A. Cersini [1], LE Bartolomé Del Pino [1,2], V. Antognetti [1], R. Lorenzetti [1], R. Nardini [1], G.L. Autorino [1], M.T. Scicluna [1]

[1] *Istituto Zooprofilattico Sperimentale Lazio e Toscana, Rome, Italy;* [2] *Complutense University of Madrid, Spain*

A molecular survey of equine piroplasms was conducted using samples collected from symptomatic and asymptomatic horses of Central Italy. Case definition for the acute form of piroplasmosis was temperature >38°C and at least one of the following signs, jaundice, anaemia and petechial haemorrhages, and a PCR positive result. Phylogenetic analysis was conducted on sequences of 78 blood samples collected in 2013/14, having either low Ct values in real-time PCRs (46 samples) for the parasites or a PCR pos/ELISA neg (VMRD, USA) result (32 samples). Sequencing was performed on the V4 hypervariable region of the 18S rRNA gene which was 390 bp for *B. caballi* and 430 for *T. equi* [1, 2]. Using BLAST, sequences were aligned with those deposited in GenBank for both piroplasms, having a minimum 98% query coverage and >97% homology. Genetic distance and homology confirmed that sequences of both parasites could be divided into the 3 groups, with a homology among the groups >97% [1, 2]. Group 1 included sequences homologous to first-ever reported piroplasms, group 2 to the "like genotypes" [1], and group 3 included those with equidistant homology for the two groups [2]. Among the 72 sequences identified as *T. equi*, 39 belonged to group 1, 24 to group 2, and 9 to group 3. Group 1 included 62% (24/39) of the sequences of symptomatic horses, while 96% (23/24) of T. equi like (group 2), were from asymptomatic horses. Studies report that sequences within group 1 and 2 are in all endemic areas, while in America only group 1 and 3 are present. To date, sequences belonging to Group 3 were never submitted for Europe and Asia. Of the 27 *T. equi* PCRpos/ELISAneg samples, 23 (85.1%) had Group 1 sequences with 19 of them from symptomatic horses. For *B. caballi*, 7 sequences were obtained, of which only 2 were from horses positive for ELISA, 4 belong to group 1, 1 to group 2, and 2 to group 3. No clinical significance was attributed to this parasite due to the limited number of sequences available. Sequences within the same groups and their wide geographic distribution suggest that the diversity could be independent from their origin and probably linked with the international movement of equidae. The present study is the first to report group 3 for both parasites

in Europe. Further studies are required to investigate the relatively low sensitivity of the ELISA and the possible correlation of the clinical evolution of the infection with the genetic group.

References

[1]. Nagore D, et al. (2004) Vet Parasitol;123:41-54.
[2]. Bhoora R, et al. (2009) Vet Parasitol;159:112-120.

205
Anthelmintic resistance in Irish equine nematode populations

N. Elghryani, T. de Waal*
University College Dublin, School of Veterinary Medicine, Dublin, Ireland

The control of nematodes in grazing equids has been reliant on the intensive use of anthelmintic drugs for many decades. This has resulted in the development of resistance against the commonly used anthelmintic drugs in many countries. We undertook a survey to investigate the prevalence of anthelmintic resistance on 14 Thoroughbred- and two donkey- populations in Ireland. Anthelmintic efficacy against nematodes was determined by calculating the percentage reduction in the faecal egg count (FEC) between the group mean at Day 0 and Day 14 post-treatment. FECs were performed using the mini-FLOTAC technique. Animals were treated with either benzimidazoles, ivermectin or moxidectin drugs. Thresholds for appropriate efficacy were chosen at an arithmetic mean FECR of >95% for ivermectin/moxidectin and >90% for benzimidazole drugs. Based on these criteria, resistance to benzimidazoles was found in 1 group of adult mares (FECR =38%) and in in three groups of yearling horses (FECR range 0-86%). Ivermectin was effective on all populations with a FECR range between 95-100%. Reduced efficacy of moxidectin was observed in one yearling group (FECR = 67%) and in two adult mare groups (FECR range 89-92%). In addition FECs were also calculated at 2 week intervals for up to 16 weeks after anthelmintic drug administration to determine the egg reappearance period (ERP) for benzimidazoles, ivermectin and moxidectin. ERP was defined when the group arithmetic mean FEC exceeded 10% of the group arithmetic mean FEC at Day 0. The results indicated that ERP for moxidectin and ivermectin ranged from 28 − 56 days and 14 days for benzimidazoles. Overall the results from this study indicate that benzimidazole, ivermectin and moxidectin are still effective on the majority of farms. However, ERP results would suggest that these products are less effective compared to label claims as a shortened ERP is believed to be an early indicator of resistance. This highlights the need for more targeted approaches in the control of nematodes in Irish equine populations to slow down the impending development of multi-drug resistance parasite populations.

Ethiopia, College of Veterinary Medicine and Agriculture, Addis Ababa University, Debre Zeit; [4] *SPANA UK, 14 John Street, London*

Histoplasma capsulatum var. farciminosum (HCF), causing Epizootic Lymphangitis (EZL), is endemic in parts of Africa including, Ethiopia, Senegal and Gambia. Despite its high prevalence, impact on animal welfare and socio-economic importance, there is little evidence upon which to build contextually relevant disease control programmes. The performance and availability of diagnostic tests currently used by clinicians is problematic. Methods such as pattern recognition of clinical signs and microscopy lack sensitivity, and other options are either not commercially available or not readily feasible (e.g. culture). This is a significant barrier to further understanding this disease within endemic countries. This study aimed to validate a nested PCR method to confirm the presence of HCF in equine clinical samples. Ethical approval was obtained from the University of Liverpool and the College of Veterinary Medicine and Agriculture. Twenty-nine horses with suspected EZL were included from topographically varied regions of Ethiopia. Clinical examination was recorded and lesion locations drawn onto equine silhouettes. Blood samples and aspirates of pus from unruptured cutaneous nodules were obtained before treatment provided by SPANA. Blood and clinical data were collected from a further 20 horses with no cutaneous EZL lesions. Giemsa stained impression smears of pus and blood were examined microscopically. Aliquots of heat-inactivated pus and blood were inoculated onto Whatman FTA cards and imported to the UK with Defra approved licensing. A nested PCR targeting the ITS region*, was used to identify samples containing HCF, and all PCR products were sequenced. HCF was confirmed in FTA card pus samples from 24 horses, additionally, 23 blood samples were positive from EZL suspected cases. The nested PCR compared favourably over microscopic examination of pus, where characteristic HCF yeast bodies were detected in only 14 of the 24 PCR positive samples examined. All nested PCR amplification products were confirmed as *Histoplasma* spp. by sequencing. Sequencing of cloned PCR amplicons suggested at least two subgroups of HCF based on single nucleotide polymorphisms. These techniques allow the rapid diagnosis of HCF directly from equine clinical samples and offer a useful epidemiological tool. The identification of HCF in blood raises questions about the pathogenesis of HCF in horses and warrants further investigation.

*Jiang, B. *et. al.* (2000) *Journal of clinical microbiology* **38** (1): 241-245.

We gratefully acknowledge funding awarded by SPANA UK, the SPANA Ethiopia team for assisting in sampling, and all participating horse-owners. Additional funds were awarded by the Institute of Infection and Global Health, and an SFAM studentship.

128
A novel molecular diagnostic tool for Equine Arteritis Virus detection and characterization

D. Gaudaire [1], N. Berthet [2], S. Zientara [3], A. Hans [1]
[1] *Anses, Laboratoire de pathologie Equine, Unité Virologie, Goustranville, France;* [2] *CIRMF, Unité Zoonose et Maladies Emergentes, BP769 Franceville, Gabon;* [3] *Anses, Université Paris-Est, Laboratoire de Santé Animale, UMR 1161 Virologie, Maisons-Alfort, France*

Equine arteritis virus (EAV) is a member of the *Arteriviridae* family. EAV infects equidae and can persist in the reproductive tract of stallions only. Stallions persistently infected shed the virus in their semen and spread the virus in the horse population during breeding. Moreover, infection may cause abortion in pregnant mares and the death of young foals. OIE prescribes viral isolation (VI) on cell culture to detect EAV for international trade. However, a recent study showed that the real-time reverse transcription-PCR (RT-qPCR) assay is as sensitive as VI for detecting EAV in semen [1]. The main challenge to EAV surveillance is detecting EAV to prevent costly outbreaks, considering in particular the limited number of viral nucleotides targeted, in some samples. The aim of our study was to increase the sensitivity of the OIE-recommended RT-qPCR method by combining it with an unbiased amplification method using the Phi29 polymerase coupled to a high-density resequencing microarray (RMA) to genotype the viruses detected. Sixty different samples were used in this study. Of the 48 EAV-positive samples, 31 were from semen, 12 were from virus isolation cell culture supernatants and five were tissue samples from the lungs, spleen or liver of one aborted foetus, three young foals and an adult. Our results showed that isothermal amplification polymerase significantly increased the ratio of amplification from a factor ranging from 10^2 to 10^7 compared to the OIE-recommended RT-qPCR method [2]. To genotype the viruses detected, we combined the unbiased amplification of nucleic acids with a RMA. The two EAV sequences tiled on the microarray cover a region located in ORF 1 coding for the non-structural protein 9. Surprisingly, the phylogenic tree obtained with the nsp 9 nucleotide sequences retrieved from the microarray was able to separate strains into the NA and EU groups and divided the EU group into subgroups EU-1 and EU-2 [2]. In conclusion, this method can be recommended for the detection of EAV in semen and aborted foetuses, especially when viral load is very low. In addition, this study confirmed and validated the usefulness of the high-density resequencing DNA microarray for both the diagnosis of equine viral diseases and the genotyping of RNA viruses such as equine arteritis virus.

1. Miszczak, F., et al., Evaluation of two magnetic-bead-based viral nucleic acid purification kits and three real-time reverse transcription-PCR reagent systems in two TaqMan assays for equine arteritis virus detection in semen. J Clin Microbiol, 2011. 49(10): p. 3694-6.
2. Hans, A., et al., Combination of an unbiased amplification method and a resequencing microarray for detecting and genotyping equine arteritis virus. J Clin Microbiol, 2015. 53(1): p. 287-91.

191
Preliminary results on the inclusion of PCR for the diagnosis of equine piroplasmosis (EP)

M.T. Scicluna [1], R. Nardini [1], L.E. Bartolomé Del Pino [1,2], A. Cersini [1], G. Manna [1], G.L. Autorino [1]
[1] *Istituto Zooprofilattico Lazio e Toscana, Rome, Italy;* [2] *Complutense University of Madrid, Spain*

EP is a tick-borne infection, caused by *T. equi* and *B. caballi*. Affected animal presents non-specific signs while introduction of the infection in free areas is due to the movement of inapparent carriers. Laboratory diagnosis of EP is generally requested for the confirmation of clinical suspects and sanitary certification of animals for trade. OIE-prescribed tests are serological methods i.e. ELISA and indirect immunofluorescence (IFAT). Even practitioners mostly request only serological tests for confirmation of suspect

[#] currently: College of Veterinary Medicine, North Carolina State University, USA

cases. In a previous study, real-time (RT) PCRs for each parasite, targeting the rRNA 18s gene (1, 2), were adopted for their optimal performance. This study presents and discusses the results of the use of RT-PCRs in conjunction with ELISA (VMRD, USA) and IFAT (Fuller Laboratories, USA) that were used to analyse 274 horse sera. For comparison of results, PCR was used as reference method since sequencing of derived amplicons confirmed them as specific. The *B. caballi* RT-PCR detected 14 positive samples, none confirmed in ELISA, while the IFAT had a sensitivity (Se) of 50% (7/14) and a specificity (Sp) of 87.7 % (228/260). The *T. equi* detected 137 positive samples with the ELISA showing a Se of 67.2% (92/137) and an Sp of 83.2% (114/137) and the IFAT, a Se of 86.1% (118/137) and an Sp of 81% (111/137). For both parasites, the IFAT presented a limited major sensitivity when compared to the ELISA. Results for *B. caballi* are preliminary as number of samples recruited is limited. In particular, for *T. equi* the serological tests showed a high agreement and a relatively high specificity. An initial evaluation of the correlation between the PCR result and clinical status of the animal was carried out defining as cases those presenting an EP acute form based on temperature >38°C and at least one of the following signs, jaundice, anaemia, and petechial haemorrhages together with a PCR positive result. Of the PCR positive horses for *B. caballi* and *T. equi*, only 28.7% and 19.7% respectively were defined as cases, possibly due to parasite persistence beyond the acute form. Seropositve results of PCR negative samples could be due to sterilization of infected horses, occurring spontaneously or following treatment with a longer persistence of antibodies as usually occurs in most infections. An additional value of the use of quantitative PCR is that treatment efficacy may be monitored especially in view of the side effects this possess. This study demonstrates that the simultaneous use of PCR with serological tests increases the diagnostic probability to define the sanitary state for EP for the purposes stated above and underlines the need for revision of the prescribed diagnostic tests for trade that should include more sensitive methods.

References

1. Bhoora R, et al. Vet Parasitol 2010;168:201-211.
2. Kim C, et al. Vet Parasitol 2008;151:158-163.

202
Validation of alphavirus chimeras for diagnosis of Eastern and Venezuelan equine encephalitis

T.L. Sturgill Samayoa*, C.D. Brotherson, K.R. Lake, E.N. Ostlund
USDA, APHIS, VS, STAS, National Veterinary Services Laboratories, National Centers for Animal Health, Ames, IA 50010

Eastern and Venezuelan equine encephalitis cause significant disease in both horses and humans.
Effective equine diagnostics (passive surveillance of cases with compatible clinical syndromes) can serve as advance warning for human illnesses. In Central and South America, there are serious difficulties in obtaining quality reagents for EEE and VEE diagnostics (per Dr. Alfonso Clavijo, Pan American Health Organization (PAHO), personal communication). Utilizing the genome of Sindbis virus (SINV), a relatively nonpathogenic human alphavirus, as a vector, a chimeric SIND/VEE virus expressing all of the structural proteins of the VEE TC-83 vaccine strain has been developed (Paessler 2003, Wang 2007). The nonstructural protein genes and cis-acting RNA genome elements from wild-type SINV strain AR339, and the structural protein genes from either North American EEEV strain FL93- 939, or from the naturally murine-attenuated SA strain BeAr436087, are included in the SIND/EEE chimeras. In collaboration with PAHO, MIDA, and the Instituto GORGAS, the National Veterinary Services Laboratories have acquired these Sindbis/Alphavirus chimeras from Dr. Scott Weaver of the University of Texas Medical Branch to validate proposed diagnostic assays. Validation of diagnostic tests that can be performed at biosafety level-2 will be beneficial for use in both the United States and in other countries. Chimeric virus propagation was performed. Direct comparison of the SIND/EEE chimera to wild-type EEE virus was performed using OIE approved protocols for EEE IgM Enzyme-linked Immunosorbent Assay (ELISA) and Plaque Reduction Neutralization Test (PRNT). Testing was performed utilizing sera from naturally infected horses, determined to be positive by previous diagnostic testing. With the goal of developing inexpensive, easily performed diagnostics, the chimeras were tested without inactivation, using the methods previously mentioned. Antigen at a 1:10 dilution provided consistently valid results with varying concentrations of both conjugate and serum. Additional testing of inactivated chimeras will also be performed. In addition to the increased safety to humans who are conducting the diagnostic testing, proposed use of chimeric strains in diagnostics will improve the ability of North, South and Central American laboratories to easily and inexpensively detect infection in animals, thereby leading to improved prevention of infection in both humans and other equids.

References

Paessler S1, Fayzulin RZ, Anishchenko M, Greene IP, Weaver SC, Frolov I. (2003) Recombinant sindbis/Venezuelan equine encephalitis virus is highly attenuated and immunogenic. J Virol. Sep;77(17):9278-86.
Wang E1, Petrakova O, Adams AP, Aguilar PV, Kang W, Paessler S, Volk SM, Frolov I, Weaver SC. (2007) Chimeric Sindbis/eastern equine encephalitis vaccine candidates are highly attenuated and immunogenic in mice. Vaccine. Oct 23;25(43):7573-81.

097
Development of a real-time PCR assay for quantification of equine herpesvirus 5 (EHV-5) and studying EHV-5 pathogenesis

L.M. Zarski, E.A. High, R.K. Nelli, S.R. Bolin, K. Williams, G Soboll Hussey*
Dpt. Pathobiology and Diagnostic Investigation, Michigan State University, East Lansing, MI, USA

Equine herpesvirus 5 (EHV-5) infection has recently been associated with equine multinodular pulmonary fibrosis in horses. To more completely understand EHV-5 pathogenesis and determine viral and host contributions, further *in vivo* and *in vitro* studies are needed. However, current techniques are unable to rapidly, specifically, and quantitatively characterize EHV-5 infection. The aim of this project was to develop a TaqMan real-time PCR assay to quantify EHV-5 in clinical and cell culture samples, and use this test to describe viral replication over time in primary equine respiratory epithelial cells (EREC)s. Primers and a probe were designed to target gene E11 of EHV-5 for TaqMan real-time PCR. Specificity was verified by testing multiple isolates of EHV-5, as well as DNA from other equine herpesviruses. A plasmid containing the target DNA was generated to create a standard curve and quantify viral copy number. TaqMan real-time PCR was performed on DNA isolated from clinical samples. In addition, ERECs were inoculated with EHV-5, and cells and supernatants were collected daily for 12 days following inoculation and TaqMan real-time PCR was performed to asses EHV-5 infection and replication.

UNIVERSITÀ DEGLI STUDI DI BARI ALDO MORO

SOIPA
Società Italiana di Parassitologia

EVPC
European Veterinary Parasitology College

XXIX CONGRESS

SoIPa

Società Italiana di Parassitologia

&

European Veterinary Parasitology College

Parasites, Poverty and Social commitment

Bari, June 21-24, 2016

Università degli Studi di Bari & Palace Hotel

FOODFOR GOOD
from meetings to solidarity

SOCIAL COMMITMENT OF THE MEETING INDUSTRY

www.soipabari2016.it

PREVALENCE OF EQUINE PIROPLASMOSIS IN CENTRAL SPAIN

Leticia E. Bartolomé del Pino, Miguel Llorens-Picher, Aránzazu Meana-Mañes

Animal Health Department, Complutense University of Madrid, Spain

Equine piroplasmosis is a tickborn disease subject to international movement regulation caused by protozoans *Babesia caballi* and *Theileria equi* that affects equidae and is endemic in many European countries. Antibodies are long-lasting (4 years for *Babesia,* lifelong for *Theileria*). To date, information on the epidemiology of equine piroplasmosis in Central Spain is limited. The aim of this study is to determine seroprevalences of both parasites and to identify associated risk factors. Equidae sera (n=88) from three asymptomatic groups in different epidemiological situations (37 rural area donkeys, 31 city area Police horses and 20 breeding facility horses) were tested using *T. equi* and *B. caballi* Antibody test kit (VMRD®). Seroprevalences were determined with exact 95% confidence levels, and compared among and within groups to evaluate risk factors (individual characteristics and management) using Chi-Square or Fishers Exact Test, whenever appropriate. P value <0.05 was considered significant (3). Overall prevalence for *T. equi* was 23,9% (95% CI: 16-34%) 13,5% donkeys, 48,4% Police and 5% breeding horses. For *B. caballi* prevalence was 5,7% (95% CI: 2-13%); 5,4%, 6,5% and 5% in donkeys, Police and breeding horses respectively. Coinfection 1,1% (95% CI:0-6%). Prevalences except for the Police group are lower than those described by other authors (1,2). Differences related to the studied risk factors were not significant, except within the donkey group where age was significant. In conclusion, similar low prevalences of *Babesia* are observed in all groups while *Theileria* was higher detected in older animals, except in the breeding farm, where more studies are needed on animal characteristics, management practices and vectors presence.

PROCEEDINGS OF THE

XVII[TH] INTERNATIONAL SYMPOSIUM OF THE WORLD ASSOCIATION OF VETERINARY LABORATORY DIAGNOSTICIANS (WAVLD)

WAVLD[17]

Saskatoon, Canada

June 15-18, 2015

PRESENTED BY THE CANADIAN ANIMAL HEALTH LABORATORIANS NETWORK (CAHLN)

047 Addition of PCR methods to conventional serology for the routine diagnosis of equine piroplasmosis

Maria Teresa Scicluna, *Roberto Nardi, Leticia Elisa Bartolomé del Pino, Ida Ricci, Francesca Rosone, Gian Luca*

Istituto Zooprofillatico Sperimentale delle Regioni Lazio e Toscana, Rome, Italy (Scicluna teresa.scicluna@izslt.it ; Nardini, Bartolomé del Pino, Ricci, Rosone, Autorino)

Equine piroplasmosis is a tick-borne protozoal infection generally difficult to diagnose clinically as clinical signs are non-specific. The infection also represents an important constraint to the international movement of equids. Common practice is the sole use of serologic tests for piroplasmosis, and for those available there is limited data on their validation. Reliable laboratory tests or their combination are essential. In a previous comparative study, real-time (RT) PCRs targeting the rRNA 18s gene (1,2), 1 for each parasite, were adopted on the basis of their optimal performance. This study presents and discusses their use in conjunction with the OIE-prescribed serologic tests: ELISA (VMRD, USA) and indirect immunofluorescence (IFAT, Fuller Laboratories, USA) to analyze blood samples from 274 horses. For comparison of the results, the PCR was used as the reference method since sequencing of derived amplicons confirmed these as *T. equi* and *B. caballi*. Case definition for an acute form of piroplasmosis was temperature >38°C and at least one of the following signs, jaundice, anemia, and petechial hemorrhages, and a PCR positive (+) result.

The *B. caballi* RT-PCR detected 14 + samples, none confirmed in ELISA, while the IFAT had a sensitivity (Se) of 50% (7/14) and a specificity (Sp) of 87.7 % (228/260). The *T. equi* RT-PCR detected 137 + samples with the ELISA showing a Se of 67.2% (92/137) and a Sp of 83.2% (114/137) and the IFAT, a Se of 86.1% (118/137) and a Sp of 81% (111/137). For both parasites, the IFAT, even if still limited, presented an apparently major sensitivity when compared to the ELISA, but liable to cross-reactivity. Results for *B. caballi* are preliminary, due to the limited number of + samples recruited.

Of the PCR + horses for *B. caballi* and *T. equi*, only 28.7% and 19.7% respectively were defined as cases, possibly due to the persistence of parasites beyond the acute form. In particular, for T. equi the serological tests showed a high agreement and a relatively high specificity. In this circumstance, the PCR negative samples could be due to the sterilization of the infected horses, occurring spontaneously or following treatment. With the further introduction of quantitative PCR, treatment efficacy could be monitored especially in view of the side effects these possess. **This study demonstrates that the simultaneous use of PCR with serologic tests increases the diagnostic probability to define the sanitary state for equine piroplasmosis for the purposes stated above.**

References

1. Bhoora R, et al. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. Vet Parasitol 2010;168:201-211.
2. Kim C, et al. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. Vet Parasitol 2008;151:158-163.

062 Preliminary observations on the genetic heterogeneity of *Theileria equi* and *Babesia caballi* in the horse population of central Italy

Antonella Cersini, *Maria Teresa Scicluna, Leticia Elisa Bartolomé del Pino, Roberto Nardini, Raffaela Conti, Giuseppe Manna, Gian Luca Autorino*

Istituto Zooprofillatico Sperimentale delle Regioni Lazio e Toscana, Rome, Italy (Cersini antonella.cersini@izslt.it ; Scicluna, Bartolomé del Pino, Nardini, Conti, Manna, Autorino)

T. equi and *B. caballi* are distributed worldwide, except for Australia. Phylogenetic analysis was conducted on sequences of 78 positive blood samples collected in 2013/14, having either low Ct values in real-time PCRs (46 samples) for the parasites (1, 2) or PCR pos/ELISA neg (VMRD, USA) results (32 samples), from symptomatic and asymptomatic horses of central Italy. Case definition for the acute form of piroplasmosis was temperature >38°C and at least one of the following signs, jaundice, anemia and petechial hemorrhages, and a PCR positive (+) result. Sequencing was performed on the V4 hypervariable region of the 18S rRNA gene (390 bp for *B. caballi* and 430 for *T. equi*). Using BLAST, sequences were aligned with those deposited in GenBank for both piroplasms, having a minimum 98% query coverage and > 97% homology.

Genetic distance and homology confirmed that sequences of both parasites could be divided into the 3 known groups (homology groups>97%). Group 1 included sequences homologous to first-ever reported piroplasms, group 2 to the “like genotypes”1, and group 3 included those with equidistant homology for the two groups (2). Among the 72 sequences for T. equi, 39 belonged to group 1, 24 to group 2, and 9 to group 3. Group 1 included 62% (24/39) of the sequences of symptomatic horses, while in group 2 96% (23/24) were from asymptomatic horses. Studies report that sequences within group 1 and 2 are in all endemic areas, while group 1 and 3 only in America. Group 3 was never submitted for Europe and Asia. Of the 27 horses, *T. equi* PCRpos/ELISAneg, 23 (85.1%) had group 1 sequences with 19 of them symptomatic. For *B. caballi*, 7 sequences were obtained, of which 2 were positive for ELISA, 4 belong to group 1, 1 to group 2, and 2 to group 3. No clinical significance was attributed to this parasite due to the limited number of sequences available. Sequences from group 1 and 3 are not reported for Europe and America, while group 2 is in all endemic areas. Sequences within the same groups and their wide distribution suggest that the diversity could be independent from their geographical origin and probably linked with the international movement of equids. **The present study is the first to report group 3 for both parasites in Europe.** Further studies are required to investigate the relatively low sensitivity of the ELISA and the possible correlation of the clinical evolution of the infection with the genetic group. On the basis of the above results, possible correlations between genetic grouping, clinical status and serological reactivity need to be further investigated.

References

1. Nagore D, et al. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: epidemiological survey and phylogenetic analysis. Vet Parasitol 2004;123:41-54.
2. Bhoora R, et al. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Vet Parasitol 2009;159:112-120.

3rd EAVLD CONGRESS

Pisa (Italy)
Palazzo dei Congressi
12-15 October, 2014

European Association of Veterinary Laboratory Diagnosticians

ABSTRACT BOOK and FINAL PROGRAMME

019

EVALUATION OF PCR METHODS FOR THE MOLECULAR DETECTION OF BABESIA CABALLI AND THEILERIA EQUI ON FIELD SAMPLES

Bartolomé Del Pino L.E. [2], Cersini A.[1], Scicluna M.T.[1], Nardini R.[1], Manna G.[1], Antognetti V.[1], Autorino G.L.[1]

[1]National Reference Centre for Equine Diseases (CERME), Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana ~ Rome ~ Italy, [2]Complutense University of Madrid, Madrid, Spain; National Reference Centre for Equine Diseases (CERME), Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana ~ Rome ~ Italy

Keywords: Equine piroplasmosis, Diagnosis, PCR

INTRODUCTION:

Equine piroplasmosis, a life threatening tick-borne disease caused by B. caballi and T. equi, is subject to international movement restrictions. While antibodies can be lifelong, as also for the carrier state, the OIE prescribed tests are still serologically based, even if these may be negative at the beginning of infection [5]. Also, the definition of the carrier state, employing direct methods is especially important in endemic areas for justifying and verifying the treatment efficacy due to its potential toxicity. For this, a study was conducted for the adoption of molecular methods for the diagnosis of these infections by evaluating the performance of different PCR methods, traditional and Real Time (RT), on field samples for both types of infections.

MATERIALS AND METHODS:

103 whole blood samples of clinically suspect equids, collected within a research project of the CERME, were analysed using 4 different PCRs for each protozoan. Genomic DNA was extracted using Cador Pathogen 96 QIAcube HT Kit (Qiagen®). PCR protocols were conducted as described in literature or according to instructions. For T. equi (T): end point PCR (T1) and nested-PCR (T2), targeting equine merozoite antigen (EMA) complex gene (amplicons 268bp and 102bp respectively) [1,6]; RT PCR (T3) targeting the 18S gene (81bp) [4]; Path-T. equi Genesig ® (T4) targeting EMA 1 (about 120bp). For B. caballi (B): End point PCR (B1) and nested PCR (B2), targeting rhoptry associated protein complex gene (825bp and 430bp respectively) [1,3]; RT PCR (B3) targeting the 18S gene (95bp) [2]; Path-B. caballi (Genesig ®) (B4) targeting the 18s gene (about 100bp). The specificity of discordant results was verified by sequencing. The PCR detecting the greatest number of positives was chosen for assessing relative sensitivity (rSe) and relative specificity (rSp). Agreement among the PCRs was estimated for each protozoan.

RESULTS:

Number of positives per method are as follows: for B1 (4); B2 (8); B3 (4); B4 (2); for T1 (29); T2 (29); T3 (35); T4 (27). An overall agreement of 91.3% was observed for B1 and 90.3% for T1. Table 1 reports the number of samples in agreement for 2, 3 and 4 PCRs. As B3 and T3 detected the highest number of positive samples and the discordant were specific products, they were used as reference tests to estimate the rSe and rSp, reported in Table 2.

	Number of PCRs in agreement					
	Babesia Caballi			Theileria equi		
	4	3	2	4	3	2
Positive	1	0	5	26	1	4
Negative	93	4		67	5	

Table 1: number of samples in agreement for 2, 3 and 4 PCRs for B. Caballi and T. equi

	T3	
	rSe	rSp
T1	80.00	98.53
T2	82.86	100.00
T4	77.14	100.00

	B3	
	rSe	rSp
B1	25.00	96.97
B2	50.00	93.94
B4	50.00	100.00

Table 2: rSe and rSp of PCRs against reference test

DISCUSSION AND CONCLUSIONS:

B3 and T3 were considered the best PCRs probably due to their primer efficiency and their short amplicons. Moreover, T3 primers were designed within a highly conserved region, and B3 employs an MGB probe consenting the use of shorter targets. The lower rSe of B1 and B2 could be due to the higher mutation frequency or degradation of their long targets, but recruitment of a major number of positives is necessary to verify this result. In general, a good overall agreement (>90%) for the PCRs of each parasite is observed. The major positivity in PCR for T. equi could be due to its reported marked higher parasitemia and prevalence than that of B. caballi [2,5]. Furthermore, from this preliminary study, when compared to the serological tests, the PCRs identified carriers among the seronegatives, as well as non-carriers among the seropositives. For T. equi, of the 36 PCR positive, 17 were seronegative and for B. caballi, all PCR positives were seronegative. In view of these results, B3 and T3 can be employed in routine diagnosis and developed as quantitative methods to assess correlation between parasitemia and the clinical phase of infection to aid the clinician, in deciding or verifying treatment. Moreover, it would be recommendable for international movement control to include PCR, in adjunct to sero-methods in use.

REFERENCES:

1) Battsetseg B. et al.; Int J Parasitol. 2001; 31(4):384-6. 2) Bhoora R. et al.; Vet Parasitol. 2010;168(3-4):201-11. 3) Bhoora R. et al.;Vet Parasitol. 2010;169(3-4):279-88. 4) Kim C. et al.; Vet Parasitol. 2008;151(2-4):158-63. 5) Mehlhorn H., Schein E.;Parasitol Res. 1998;84(6):467-75. 6) Nicolaiewsky T.B. et al.; Vet Parasitol. 2001;31;101(1):9-21.